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ABSTRACT OF THESIS

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Title of Thesis STUDIES OF POLYSACCHARIDES OF THE ENTEROBACTERIA

The acid-stable monosaccharide units of the E.coli K12, S53 extracellular slime have been characterised after cellulose-column chromatography of an acid hydrolysate. Quantitative analysis of the polysaccharide has indicated that fucose, glucose, galactose and glucuronic acid are present in approximate ratios of 2:2:3:2, with 10% of the polysaccharide unaccounted for at this stage.

Several methylation techniques have been employed on the polysaccharide, the most successful being the use of barium hydroxide and methyl iodide in N-methyl-2-pyrrolidone solution. Methylation was then completed by subsequent treatment with silver oxide and methyl iodide. Hydrolysis and column chromatography, followed by characterisation of the fractions, revealed the presence of 2,3-di-O-methyl glucuronic acid, 2,4,6-tri-O-methyl galactose, 2,4,6-tri-O-methyl glucose, 2,3-di-O-methyl fucose, 2-O-methyl fucose and an unidentified end-group component,X . Several polysaccharides from other strains have been shown to yield identical products upon hydrolysis of their methyl ethers.

Alkaline fragmentation of S53 polysaccharide ester, presumably by β-elimination, has resulted in the release of an acid-labile acid component. No other methods have been found for the isolation of this sugar. Methylation has shown a relationship between this monosaccharide and X. The products ~~are~~ of enzymic degradation of the polysaccharide have been shown to be of large molecular weight.

The use of a wide range of physical and chemical methods in the examination of the acid-labile monosaccharide (and its methylated derivative X) have led to a provisional structure for the sugar being suggested. A partial structure for the polysaccharide is also proposed.

Use other side if necessary.

STUDIES OF POLYSACCHARIDES OF THE ENTEROBACTERIA

By

Christopher William McCleary B.Sc.

oooOooo

Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh September, 1967.

To Carol and Paul,

and my parents

Acknowledgements

I wish to express my grateful appreciation to Professor Sir Edmund Hirst who, in addition to providing laboratory facilities, was also a source of considerable encouragement. These sentiments are also extended to Dr. John F.Wilkinson of the Department of Bacteriology who was unfortunate enough to be one of my supervisors; his forbearance was outstanding. The interest and practical assistance of Dr. Ian W.Sutherland of the same Department were similarly much appreciated.

The provision of a very generous Faculty of Medicine Research Scholarship gave me the opportunity to study for this degree, and I extend my sincere thanks for this support.

To Dr. David A.Rees I owe a debt which can never be repaid but which will always be remembered. If a small proportion of his sense of purpose and his attributes in scientific research have rubbed off onto his student, I am very fortunate. Although not mentioned under 'general methods', Dr. Rees was responsible for removing (and putting on) most of my paper chromatograms, and also for reducing the Thesis Introduction from 24 pages to 35.

That my wife completed her degree and teacher-training courses, and successfully managed a young son and a house during my studies, speaks volumes for her personal attributes. Her patience and understanding have sustained and encouraged me. My thanks also to my son, Paul, who always selected the least worthy pages of my Thesis drafts upon which to design cars or which to otherwise deface.

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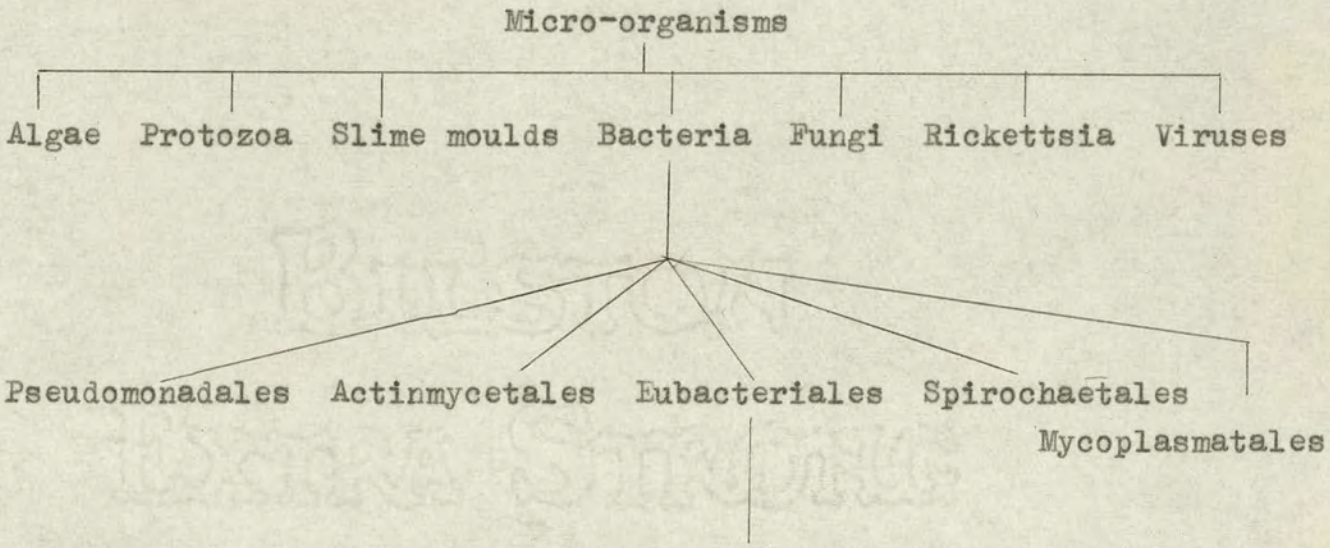
Studies on the polysaccharides of the Enterobacteria - General Introduction

1. General features of the Enterobacteria.
2. Early history of studies in bacterial polysaccharides.
3. Distribution and functions of polysaccharides in bacteria.
4. Monosaccharide units found in bacterial macromolecules.
5. Structures of some extracellular polysaccharides investigated by other workers.

1. General features of the Enterobacteriaceae

As in the animal system, micro-organisms are classified in orders, families and genera, and the diagram below gives some idea of the position of the enterobacteriaceae in relation to micro-organisms as a whole:-



9 families including the Enterobacteriaceae

The Enterobacteriaceae family constitute Gram negative, rod-shaped cells, which can grow well on ordinary peptone media and which readily ferment glucose, lactose and other sugars, producing acid or acid and visible gas. Of the Enterobacteriaceae, the Escherichia, Cloaca and Klebsiella genera are the three which are discussed in detail or in outline in subsequent chapters (reference will be made to Cloaca cloacae, which has been named such rather than Aerobacter cloacae; this conforms with recent re-classification of the previously-named Aerobacter genus). It would be impracticable

to give more than a brief summary of the characteristics of Escherischia coli, Cloaca cloacae and Klebsiella strains, and only the major, salient points are therefore outlined below:-

Escherichia coli

E.coli strains predominate among the aerobic commensal organisms present in the healthy gut. Although predominantly commensal, some O-inagglutinable strains have been incriminated as pathogens found frequently in pyogenic infections of the urinary tract, peritonitis, septic wounds and bed-sores. In the vetinary field, pathogenic strains of E.coli are responsible for 'coli-septicaemia' in calves, mastitis in cows, and a disease resembling tuberculosis in poultry.

Most strains of E.coli are flagellate and fimbriate, and a few are capsulate, thelatter strains being of particular importance in this thesis.

Cloaca cloacae

Members of the genus Cloaca are widely distributed in nature, and are commonly encountered in water and grasses. They are occasionally isolated in small numbers from healthy faeces but are not regarded as pathogenic for man or animals. Strains are seldom capsulate but are frequently motile, flagellate and fimbriate, and can be differentiated from the related Klebsiella species by their ability to liquify gelatin.

Klebsiella species

Klebsiella strains vary from harmless commensal types to pathogenic species such as Kl. pneumoniae. Microscopically, these organisms can be shown to possess large capsules or extracellular slime, causing their colonies to be mucoid or viscid; by means of the capsular antisera, the Klebsiella genus can be divided into at least 72 serotypes, the tests being performed as agglutinations or by a capsule-swelling reaction observable under the microscope.

2. Early history of studies in bacterial polysaccharides

The subject of bacterial polysaccharides dates back to the middle of the 19th century, when Louis Pasteur became the first investigator to prove that the slime 'viscous' fermentation of carbohydrate solutions was bacterial in origin. He isolated the gummy material and assigned to it an empirical formula of $C_6H_{10}O_5$. The first gum of bacterial origin to be studied comparatively carefully, however, was a dextran produced by Leuconostoc mesenteroides, a gum which was interfering with the crystallisation of sucrose during refinement. Van Tieghem (1878) gave the first adequate description of the casual organism, and a fairly complete identification and characterisation was finally effected by Lipmann in 1904. During the latter part of the 19th century, many other organisms were found to produce dextrans, Micrococcus viscosus (Bechamp 1881), Bacillus viscosus sacchari (Kramer, 1889)

and Micrococcus gummosus (Happ 1893) being but a few examples.

A range of simple polysaccharides from non-pathogens were isolated and tentatively characterised by 1920, and Tomura showed that the pathogens Corynebact. diphtheriae and Mycobact. tuberculosis contained a pentosan which liberated arabinose on acid hydrolysis.

The 1920's brought the discovery that certain carbohydrates of micro-organisms showed antigenic properties. Heidelberger and Avery (1923) observed a marked difference in the antigenic properties of an intact pneumococcus and its polysaccharide. They wrote that "the cellulose substances of pneumococcus, although lacking the specific antigen of the whole cell, induce the formation of antibodies reacting with pneunococcus protein regardless of the type from which the latter is derived". Zinsser and Takete (1924) found similar antigenic relationships of protein and carbohydrate from cells of tubercle bacilli, pneumococcus, meningococci, staphylococci and typhoid bacilli.

Thus, by 1925, basic chemical and immunochemical studies were well-established, and part 5 of this general introduction will indicate some of the more recent and detailed studies made on extracellular polysaccharides in particular.

3. Distribution and functions of polysaccharides in bacteria

The eubacterial cell is surrounded by a wall, the thickness and structure of which vary somewhat in different species; In the living cell, this uniform or multi-layered wall closely invests the cytoplasmic region which is bounded by a much thinner and more

delicate membrane, the cytoplasmic membrane. The cytoplasm itself appears fairly uniform except where it is interrupted by deposits of reserve materials, such as poly-B-hydroxybutyric acid, glycogen or volutin, (transient cytoplasmic constituents, the presence, nature and amounts of which are determined largely by environmental conditions). The nuclear region is quite uniformly filled with a very fine fibrillar network, sharply discontinuous with the surrounding ribosome-filled cytoplasm, although not separated from it by a membrane.

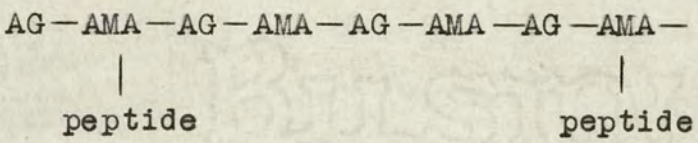
In addition to locomotor organelles, termed flagella or fimbriae, there frequently exists extracellularly a capsule or slime layer, often extending for a considerable distance into the surrounding medium.

The cell wall

The cell wall always represents a major fraction of the total material in the eubacterial cell and accounts for 20 - 35% of its dry weight. The wall has been shown to play a purely mechanical role, by restricting the enclosed protoplast to a fixed maximal volume. This prevents the uptake of water, with consequent swelling and rupture, when (as is commonly the case for bacteria) the cell exists in a strongly hypertonic solution. The wall does not seem to play any role in regulating permeability or in the direct metabolic activities of the cell.

It is apparent that there are major and consistent differences between Gram-positive and Gram-negative bacteria with respect to the nature of the macromolecular compounds that are

incorporated in the wall, the only macromolecular compound that is universally present being a glycopeptide responsible for conferring structural rigidity to the wall. The backbone of this glycopeptide is essentially a polysaccharide, consisting of alternating units of acetylglucosamine and acetylmuramic acid, while the side chains, linked through the free carboxyl group of the muramic acid, consist of short peptide chains containing only a few specific amino acids from the twenty-odd found in proteins:



AG = acetylglucosamine
AMA = acetylmuramic acid

As a general rule, the walls of Gram-positive bacteria consist predominatly of the glycopeptide. The walls of Gram-negative bacteria, however, always contain large amounts of protein, lipid and other polysaccharides, and in the case of Escherichia coli, it has been shown that the glycopeptide is associated with the inner layer of the wall, while the outer layer consists of a lipo-polysaccharide-protein complex. Both in terms of chemical composition and of microstructure, the walls of Gram-negative bacteria are thus characteristically more complex than those of Gram-positive ones.

Penicillin owes its effectiveness as an antibacterial agent to the fact that it specifically prevents the formation of peptide cross-links between the glycopeptide chains. As the cells grow in the presence of the antibiotic, the newly synthesised

regions of the wall are, as a result, deprived of their tensile strength, giving rise to bizarre, distorted cells. Gram-negative cells, with their lower mucocomplex content, are obviously much less liable to be adversely affected in the presence of penicillin.

The cytoplasmic region

In addition to the cell membrane, which contains lipid and protein and is closely associated with the cell-wall in Gram-negative bacteria, the cytoplasm contains the protein-synthesising ribosomes and cellular reserve materials. In general terms, the latter consist of poly B-hydroxybutyric acid, polymerised inorganic metaphosphate (volutin), glycogen or elemental sulphur (e.g. thiothrix). In coliform bacteria, the sole energy-reserve material is glycogen. A massive intracellular accumulation of glycogen occurs characteristically when the cells are provided with a source of carbon and energy in the absence of a nitrogen source essential for protein synthesis. Reserve polymer may be formed so massively that it amounts to 50% of the dry weight of the cell. If such cells are then provided with an external source of nitrogen, the storage polymer is degraded and used as a source of carbon and energy for general cellular synthesis.

Capsules and slime layers

Many bacteria secrete organic polymers with a limited solubility in water which tend to accumulate as loose, confluent layers in the immediate neighbourhood of the cells, just outside the wall. These structures are commonly referred to as capsules,

if they are relatively small and sharply delimited, and as slime layers or slimes, if they are more extensive and diffuse.

Most commonly they are polysaccharides, but the capsular substances of a few Gram-positive bacteria are simply polypeptides composed of glutamic acid bonded through the terminal carboxyl group instead of the a-carboxyl group as in proteins. Acetobacterxylinum has an unusual slime layer, consisting of cellulose deposited as a loose fibrillar network between the cells. More commonly, however, the capsular polysaccharides are composed of sugars, amino sugars, and uronic acids, related polysaccharides often displaying a very similar ratio of constituent sugars. Capsules apparently identical chemically may display considerable antigenic specificity. Such specificity has been studied in great detail in the Klebsiella and Pneumococcus, the latter being the principal causative agent of bacterial pneumonia; over thirty different strain-specific, pneumococcal, capsular polysaccharides have been discovered.

Unlike the cell-wall, the capsule or slime layer does not seem to have a direct role in the maintenance of cellular function. Some streptococci and bacilli, for instance, only forming a capsule when growing at the expense of a specific substrate which is a direct biochemical precursor of the capsular substance in question. These organisms form copious quantities of either dextrans or fructans when growing at the expense of the disaccharide sucrose; no other metabolisable sugar, including glucose and fructose themselves can serve as a substrate for the synthesis of these polysaccharides.

Another observation is that the ability to form a capsule can be lost as a result of mutation, as in the pneumococcus and a number of other bacterial species, and in no case does it have a deleterious effect on bacterial growth under pure culture conditions. Further, certain kinds of capsular substances can be removed from the cells by treatment with the specific hydrolytic enzymes; such enzymatic treatment leaves the cells unharmed.

Although the facts outlined above show that the capsules and slime layers are not essential components of the cell, the very widespread ability among eubacteria to synthesise these structures suggests that they may have considerable survival value under the natural conditions of bacterial existence. In certain species, for example, only capsulated strains are capable of being pathogenic, capsule-free mutants derived from these virulent strains being essentially non-pathogenic. The presence of a capsule makes bacterial cells less susceptible to engulfment and subsequent destruction by the phagocytes of the host animal. Free-living bacteria may be similarly protected against phagocytic engulfment by protozoa. A further function of the capsule may be protection against viruses. In order to attack a bacterium, the virus must attach itself to the cell-wall as a preliminary step, and the presence of copious slime or a capsule must obviously provide the bacterium with a measure of protection against attack of this kind.

<u>The antigenic function of polysaccharides of the enterobacteria</u>

When certain foreign materials of high molecular weight

are injected into the body of an animal, substances appear in the serum which are capable of specific combination with the injected material. The substance appearing in the serum is called an antibody; the materials capable of evoking anitbody formation are called antigens. It has long been known that bacteria, even closely related ones, can be distinguished from one another by their antigenic specificity, as determined by suitable immonological procedures. Antigenic analysis is in fact by far the most sensitive instrument for the detection of slight differences between bacteria, and has great practical importance in diagnostic medical bacteriology and epidemiology.

Basically, the antigenic specificity of bacteria is an expression of their specific surface structures. While the capsular or slime K- antigens are somewhat likely to cause difficulties in serological identification of the bacteria by masking the O-antigen of the cell wall, the capsule appears to have considerable immunological importance from an infection standpoint. As has already been mentioned, freshly isolated pneumococcus and typhoid bacillus possess a capsular antigen associated with virulence. When the organism is continuously cultivated artificially and loses its virulence, this antigen is no longer present (the presence being detected by specific agglutination tests), and such antigens are termed Vi (virulence) types.

It is clear that polysaccharides in bacteria are of major importance, both in a subjective functional sense and also in

direct serological activity *in vivo*. The determination of structures of related bacterial polysaccharides would be an obvious preliminary step towards accounting for the remarkedly unique characteristics that these polysaccharides possess.

4. Monosaccharide units occurring in bacterial macromolecules

It will be shown in the later part of this thesis that some of the polysaccharides examined contain an unusual and hitherto unreported monosaccharide component. As a background to this discovery, a table of known monosaccharide components found in bacterial polysaccharides has been compiled, and is included in this section. Such a list is not intended to be an exhaustive survey on bacterial sugars, but rather a broad summary of the main types.

The review by Stacey and Barker (1) and the subsequent comprehensive survey of pneumoccocal polysaccharides (2) have shown that certain neutral sugars, common throughout nature, are similarly much in evidence in bacteria. In addition to the sugars listed in Table 1, ribitol is also present in bacterial macromolecules (3). It is not proposed to elaborate on the chemistry of these simple neutral sugars since they form the basic text of any elementary textbook on carbohydrate chemistry, and we will accordingly concentrate on the slightly less common sugars of less universal distribution.

a) 2-amino-2-deoxy sugars

In addition to glucosamine and galactosamine which occur

widely elsewhere in nature, bacterial polysaccharides have been found to contain D- and L-fucosamine and pneumosamine, the last-named (talosamine) having been detected in only one instance, the pneumococcus Type V polysaccharide.

The polar amino group lends itself readily to the use of ion-exchange chromatography for the separation of the amino-sugars from complex mixtures in hydrolysates, and their comparative stability in acid solution together with their formation of salts with inorganic acids makes their detection, isolation and purification a comparatively facile procedure. While normally detected in biochemical laboratories by the Elson-Morgan colorimetric method (4), crystalline derivatives are readily formed (phenyl isocyanate and carbobenzoxy derivatives) for characterisation purposes. A useful specific reaction of 2- amino sugars is the shortening of the carbon chain to yield nitrogen-free lower aldoses by oxidation with ninhydrin or hypochlorite, glucosamine and galactosamine being converted to arabinose and lyxose respectively. Kuhn (13) has used a reversal of this reaction to synthesise these and other amino sugars.

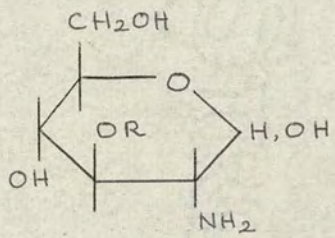
The group of natural amino-sugars comprises some less common esoteric entities. Cultures of Aerobacter aerogenes and E.coli synthesise 1-(O-carboxyphenylamino)-1-deoxy-D-ribulose, while Salmonella typhimurium hydrolysates yield 1-(O-carboxyphenyl)-D-fructosamine, the structures of which are indicated in the Table.

Table 1 Monosaccharide units occurring in bacterial macromolecules

Type of sugar	name	examples of occurrence	reference
1. Common neutral sugars			
	D-glucose D-galactopyranose D-galactofuranose D-rhamnose D-mannose L-fucose L-rhamnose	Widespread occurrence in Gram -ve and Gram + ve bacteria	
	D-ribose Deoxyribose	RNA and DNA	
2. Other sugars			
a) Aminosugars	D-glucosamine	Lipopolysaccharides of Chromobacterium violaceum	5
		Bacillus subtilis	6
		and Micrococcus lysodeikticus	7
	D-galactosamine	Cell-wall glycopeptide with muramic acid	
		Lipopolysaccharide of Bacillus subtilis	6
	D-fucosamine	Lipopolysaccharide of Chromobacterium violaceum	8 9
	L-fucosamine	Pneumococcus Type V capsular polysaccharide	10
	Pneumosamine (2-amino-2,6-dideoxy-L-talose)		

Table (continued)

Type of sugar	name	examples of occurrence	reference
	1-(O-carboxyphenylamino)-1-deoxy-D-ribulose	Cultures of Aerobacter aerogenes and of E.coli.	11
	1-(O-carboxyphenyl)-D-fructosamine	cultures of Salmonella typhimurium	12
b) amino-acid sugars	(i) muramic acid	the cell-wall glycopeptide in most bacteria	14



R = - CH(CH3)COOH

(3-O-(α-carboxyethyl)-D-glucosamine

	(ii) nonulosaminic acid (N-acetyl N-O-diacetyl)	colominic acid from E.coli	15
		E.coli endotoxin	16

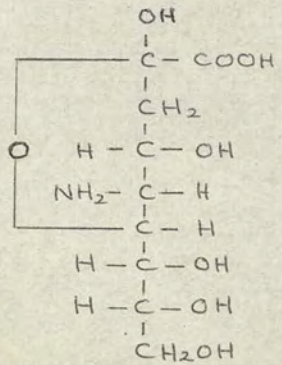
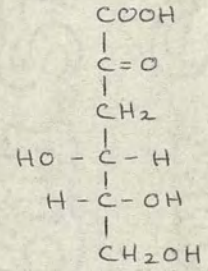
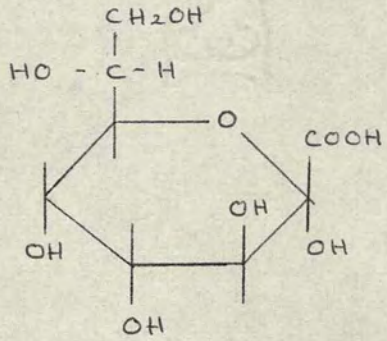


Table 1 (continued)

Type of sugar	name	examples of occurrence	reference
c) Deoxy sugars			
	L-fucose L-rhamnose	wide general occurrence	
	D-rhamnose and 6-deoxy-D-talose	a soil bacterium, 'GS'	20
	L-talomethylose	cell walls of Mycobacterium marianum	
	2-O-methyl L-fucose and methyl ethers of L-rhamnose	polysaccharides of Mycobacterium tuberculosis	21
d) Dideoxysugars			
abequose (3-deoxy-D-fucose)		polysaccharides of Salmonella Typhosa	22
Tyvelose (3-deoxy-D-rhamnose)		" " Salmonella salinatis	23
ascarylose (3-deoxy-L-rhamnose		" " P.pseudotuberculosis	24
paratose (3,6-dideoxyribohexose)		" "Salmonella paratyphii	25
colitose (3-deoxy-L-fucose)		the 'O' antigen of an E.coli	26
e) Uronic acids			
	D-glucuronic acid D-mannuronic acid D-galacturonic acid	Wide general occurrence, especially in extracellular polysaccharides	
	L-guluronic acid	Extracellular polysaccharides of Pseudomonas aeroginosa and Azotobacter Vinelandii	27 28
	D-galactosaminuronic acid	Vi antigen of an E.coli	29
	D-mannosaminuronic acid	Cell walls of M. lysodeikticus	30

Table 1 (continued)

Type of sugar	name	examples of occurrence	reference
f) Higher-carbon sugars			
	D-glycero-L-manno-heptose	Shigella flexneri	32
	Dglycero-D-galacto-heptose	Chromobacterium violaceum	33
	L-glycero-D-manno-heptose	E.coli cell walls and	34
	D-glycero-D-manno-heptose	extracellular polysaccharide of S. marcescens	35
g) Other monosaccharides			
	(i) 2-keto-3-deoxygalactonic acid 	Extracellular polysaccharide of Azotobacter vinelandii	37
	(ii) 3-keto-2-deoxy-manno-octonic acid 	Many bacterial lipo-polysaccharides	38

b) Amino-acid sugars

The most important amino-acid sugar found in bacterial macromolecules is the compound muramic acid. With acetyl glucosamine, this is a major component of the cell-wall glycopeptide, the prime function of which is to give some measure of structural rigidity to the cell. First isolated in 1954, it was identified as 3-O-(α-carboxyethyl)-D-glucosamine (7,8). Purification was effected on ion-exchange columns after prior removal of glucosamine, and the material treated with hydriodic acid and red phophorus to detect an alkyl iodide which might indicate an alkyl side-chain. While this was found to be absent, a propionic acid residue was detected, and on the basis of periodate studies and Elson-Morgan reactions, it was concluded that the original substituent was an O-α-carboxyethyl group and that this was attached to a hexosamine via an ether linkage through position 3. Synthesis was effected commencing with N-acetyl-4:6-O-benzylidene-α-methyl-D-glucosaminide and using sodium hydride to form the 3-sodio derivative which was then reacted with ethyl-α-DL-iodo-orthopropionate. The two possible isomers were separated by virtue of their solubility characteristics, and the desired isomer hydrolysed to yield a product which was identical upon crystallisation with naturally-occurring muramic acid.

A range of sugars found widely in mammalian tissues but less commonly in bacteria are the sialic or nonulosaminic acids, these being O- and N-acetyl derivatives of a 9-carbon sugar, neuraminic acid. N-acetyl neuraminic acid constitutes the

monomer unit in the polymer colominic acid, (15) and Dewitt and Rowe found N-acetyl and N-O-diacetylneuraminic acid in crude mixtures from the endotoxin of E.coli; it was later shown by Dewitt and Zel (16) that these acids were accociated with the extreme-surface lipoprotein layer of the cell, and were not constituents of the cell wall itself.

While various colour reactions had given an indication of possible structures for neuraminic acid, more direct evidence came from the discovery that the action of alkali gives rise to pyrrole-2-carboxylic acid, and Gottschalk inferred that this and previous information indicated a compound which could be envisaged as the result of aldol condensation of N-acetyl-D-glucosamine with pyruvic acid. Synthesis of N-acetylneuraminic acid was effected by condensing N-acetylglucosamine with oxalacetic acid at pH 10-11 (17), but subsequent workers (18) showed that enzymic cleavage of the neuraminic acid produced N-acetylmannosamine, a reaction which was reversible, and that the synthesis outlined above was the result of easy epimerisation of N-acetylglucosamine in the alkaline solution. The configuration at C_4 had been given as being L- on account of a high negative rotation of the γ-lactone, but was shown to be in fact D- (19) by degradation of a desthiolactone derivative of N-acetylneuraminic acid to R-(-)-pentane-1,4,5-triol, the latter being unambigusously synthesised by 3 different methods;

In addition to their lability to alkali, nonulosaminic acids appear to be very acid-labile, giving rise to intractible material.

c) Deoxy sugars

As well as L-rhamnose and L-fucose already mentioned most of the bacterial deoxy-sugars are of the 6-deoxy type, D-rhamnose, 6-deoxy-D-talose, L-talomethylose and 2-O-methyl-L-fucose having been isolated (see Table 1). Synthesis is usually effected by removal of a 6-tosyloxy group, either directly with $LiAlH_4$ or indirectly by replacement of the group with an iodine atom followed by reduction with $LiAlH_4$ or raney nickel. Unlike 2-deoxy sugars, the 6-deoxy type are comparatively stable in acid solution; colour reactions are also very similar to those of hexoses or pentoses, and the wide range of colour reactions used to detect the 2-deoxy sugars are not applicable to the 6-deoxy type.

d) Dideoxy sugars

An increasingly large number of dideoxy sugars is being found in the bacterial endotoxin lipopolysaccharides, all at present being 3,6-dideoxy sugars. 5 of 8 possible dideoxyhexoses have been identified, abequose (3-deoxy-D-fucose), tyvelose (3-deoxy-D-rhamnose, ascarylose (3-deoxy-L-rhamnose), paratose (3,6 dideoxy-hexose) and colitose (3-deoxy-L-fucose). The 3,6 dideoxyhexoses have considerable biological importance as they have been shown to contribute to serological specificity of many immunologically active polysaccharides, and the liberation of these sugars by

treatment with dilute acid for 5 minutes at 100°C while the rest of the polymer remains intact indicates that they are probably non-reducing end-groups.

e) Uronic acids

The D-series of glucuronic, mannuronic and galacturonic acids are of wide and general distribution, especially in extracellular polysaccharides. More recently, the extracellular polysaccharide of Azotobacter vinelandii has been found to closely resemble alginic acid and to accordingly contain large quantities of guluronic acid. Pseudomonas aeroginosa strains yield polysaccharides with varying proportions of guluronic acid. It is possible that several other polysaccharides believed to contain mannuronic acid may also contain guluronic acid if research was conducted on these polysaccharides prior to the advent of chromatographic techniques suitable for separating these sugars.

The comparative strength of uronic acid glycosidic linkages results in the necessity of using 4% sulphuric acid for 24 hours at 100°C to effect substantial hydrolysis of these linkages. Such reaction conditions result in appreciable decarboxylation. Another feature of uronic acids is the ease with which they can be epimerised at C-5 to interconvert acids of the D-series and this being accomplished in neutral solution at 100°C; the products are separated from the parent acids on ion exchange columns.

In addition to the straightforward uronic acids, an amino-deoxyuronic acid has been found in the Vi antigen of E.coli (29) and was subsequently identified as D-galactosaminuronic acid

(see Table 1). More recently, H.R. Perkins (30) used trichloroacetic acid to extract from the cell walls of M. lysodeikticus a polymer consisting of glucose and acetamidomannuronic acid. The latter was found to be considerably labile to acid hydrolysis with the production of ammonia, and it was concluded that the compound is N-acetylated thus facilitating hydrolysis of what might otherwise be an acid-stable glycosidic linkage. Degradation of the liberated monomer then occurs.

f) Higher-carbon sugars

While heptoses, heptuloses, octuloses and even nonuloses have been found in the plant kingdom, the higher-carbon sugars found in bacterial polysaccharides appear to have been confined to aldoheptoses, and to date such sugars are only evident in Gram-negative bacteria, generally as components of polysaccharides which determine the immunological specificity of the cells (31). These polysaccharides contain a variety of constituent sugars including the dideoxy hexoses already discussed. Actual identification of aldoheptoses has been effected in only a limited number of cases, examples of which are given in Table 1. Most reports, however, concern L-glycero-D-manno-heptose, the heptose contents of polysaccharides varying from more than 50% in Pasteurella pestis to only 5% in some strains of P. pseudotuberculosis (35). More recently, Young and Adams (36) have shown that an extracellular polysaccharide from Serratia marsescens contain D-glycero-D-manno-heptose and D-glucose as main-chain units, the chains being

terminated by L-glycero-D-manno-heptose, and to date this polysaccharide appears to be unique in this unusual constitution.

While identification of aldoheptoses is generally effected by formation of a crystalline derivative as for the common hexoses, preliminary identification can frequently be effected by brief periodate oxidation of the intact polysaccharide, followed by reduction with sodium borohydride and acid hydrolysis. The heptose (e.g. D-glycero-D-manno-heptose) is replaced in acid hydrolysates by a hexose (D-mannose), indicating the presence of a manno-heptopyranose having unsubstituted hydroxyl groups at C_6 and C_7.

Certain aldoheptoses form anhydrides under acid conditions, the altro- gulo- and ido-configurations doing so particularly readily. This has been attributed to the stability of the β -D-anomers in the 1C configuration. Heptoses behave towards strong acids in a manner closely related to that of hexoses or pentoses, and can be identified as heptoses in suitable colour reactions such as the cysteine-sulphuric acid test.

g) Other monosaccharide constituents

Claus (37) has identified an unusual component of an extracellular polysaccharide of Azotobacter vinelandii as 2-keto-3-deoxygalactonic acid. In a polysaccharide consisting largely of rhamnose, the ketodeoxy acid appears to constitute a non-reducing end-group. Prior to Claus's work, the acid had only been reported as an intermediate in the metabolism of galactose and

galactonic acid in Pseudomonas saccharophila and Gluconobacter liquefaciens (37a). Isolated from acid hydrolysates of the polysaccharide, the substance reacted with o-phenylenediamine to give a compound which had absorption characteristics similar to that given by the reaction of an α-ketoacid with o-phenylenediamine. Reaction with thiobarbituric acid indicated the presence of a 2-keto-3-deoxyaldonic acid, and decarboxylation gave CO_2 corresponding to one carboxyl group. Consumption of periodate and production of formaldehyde was examined quantitatively, and it was concluded that 3 adjacent hydroxyl groups were present, one of which was terminal. The rate of periodate oxidation indicated the galactose-type disposition of hydroxyls on C_3-C_4 as opposed to the possible glucose-type configuration. Chromatographic mobility was found to be identical with that of synthetic material prepared from galactose.

While 2-keto-3-deoxy arabonic, 2-keto-3-deoxy gluconic and a 2-keto-3-deoxy heptonic acid have all been reported as metabolites, their incorporation in polysaccharides has yet to be described.

At the octose level, however, Levin and Racker (39) described the properties of an enzyme capable of synthesising a 2-keto-3-deoxy-octonic acid phosphate (KDO phosphate), synthesis of hexonic and heptonic acids by an analogous reaction having previously been reported. The presence of KDO in polysaccharide material was detected, by Heath and Ghalambor (38), in the lipopolysaccharide of an E.coli strain, and it has since become

apparent that the KDO has an important significance in the lipopolysaccharide structure. It has been shown (40) that KDO is the component of the lipolysaccharide 'core' involved in the covalent linkage between the polysaccharide and the lipid. Little is known of the polysaccharide core although Heath and Edstrom (41) have demonstrated the enzymic incorporation of this substance in cell-free systems using CMP - KDO with alkali-treated, acid degraded lipopolysaccharide (lacking KDO) as acceptor.

The structure of KDO from lipopolysaccharides was examined (38) using colorimetric, periodate, oxidative decarboxylation and chromatographic techniques on the material isolated from the lipopolysaccharide, and on synthetic KDO, using ketodeoxygluconate and ketodeoxyheptulosonate as comparisons. They concluded that the lipopolysaccharide KDO was either identical to synthetic KDO or was its enantiomorph.

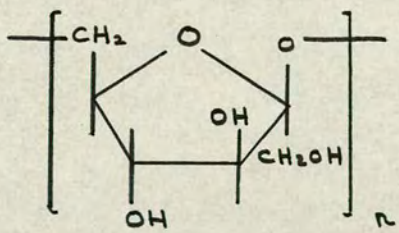
The configuration of the hydroxyl groups in the molecule was established by the tentative identification of 2-deoxy-D-mannoheptose produced by ceric sulphate oxidation of the reduced 3-deoxyoctulosonic acid (42), and the synthesis of this 2-deoxy-D-mannoheptose has been reported by Perry (43). 1-deoxy-1-nitro-D-glycero-D-galacto-heptitol was acetylated and then treated with sodium bicarbonate to yield 3,4,5,6,7-pentaacetoxy-D-manno-1-nitro-1-heptene which was reduced to the 1,2-dideoxy heptitol. Removal of the acetyl groups with alkali followed by treatment with sulphuric acid gave 2-deoxy-D-mannoheptose.

5. Structures of extracellular polysaccharides investigated by other workers

Extracellular polysaccharides elaborated by bacteria are both great in number and diverse in nature, the Pneumococcae alone being capable of producing over seventy different types of capsule as determined by serological techniques. To comprehensively summarise the research into the structures of these polysaccharides is an attainment beyond the limits of this thesis, but typical examples from Gram-negative and Gram-positive bacteria will be used as general illustrations. Some polysaccharides which have been more exhaustively examined with chemical and enzymic methods will be discussed at length.

A) Homopolysaccharides

Extracellular bacterial levans and glucans are well known and identified and will be only briefly discussed here. Many Pseudomonas, Xanthomonas and Bacillus species produce copious quantities of levans of the β-2,6-type



The structure of these polysaccharides is of historical interest, the elucidation of the main features being effected by the hydrolysis of the methylated polysaccharide and identification of the 1,3,4,6 tetra-O-methyl and 1,3,4 tri-O-methyl D-fructoses.

The production of cellulose by species of acetobacter and of dextran by Leuconostoc is also well documented. Both were examined in the 1930's using methylation and acetolysis techniques, and, in the case of cellulose, by X-ray diffraction. The synthesis of dextran by the Leuconostoc group has been summarised in a review by Stacey and Ricketts (44), the chief interest in dextran being its use as a plasma-substitute, although dextran sulphate has also been used as an anti-coagulant for certain blood disorders. Much of the subsequent work on bacterial dextrans has been related to the incidence of 1,2- linkages in the polysaccharide, and also to the chain lengths, which vary from 5-40 units).

As well as being noted for levan production, the Bacillus genus includes the species B.macerans which has long been known to elaborate non-reducing crystalline saccharides when grown on a starch medium. Investigation has shown that these Schardinger dextrins are cycloamyloses in which 6,7 or even 8 glucose units are joined by α-1:4-linkages to form a continuous loop (45). It is now recognised that the enzymic transfer of α-1:4-linked glucose units from amylose to form cyclic dextrins is facilitated by the natural tendency for chains of α-1:4-glucose units to assume a helical conformation.

Various strains of Agrobacterium have been shown to elaborate a glucan (46); periodate oxidation of this and of the sodium borohydride reduction product has indicated a linear 1,2-glucopyranose structure, and optical rotation on the poly-

saccharide and oligosaccharides obtained by partical hydrolysis has indicated a β-configuration for the glycosidic linkages. The partial hydrolysis yielded oligosaccharides with chain lengths of 2 to 6, these being characterised chromatographically and by the action of periodate. Methylation was less useful here than is normally the case owing to a failure to substitute some of the C_3 hydroxyls, a failure which is probably on account of hydrogen bonding with the ring oxygen of the adjacent pyranose unit.

More recently, Chondrococcus columnaris has been shown to elaborate a high molecular weight homopolymer consisting of D-galactosamine (47), in which half the amino groups are acetylated (as shown by analysis and by proton magnetic resonance and infra-red spectroscopy). Consumption of 0.6 moles of periodate per sugar unit was attributed to 1,4 linkages in which half the galactosamine residues were N-acetylated, and the glycosidic configurations were given as α on the basis of optical rotation and proton magnetic resonance spectroscopy.

B) Heteropolysaccharides

The immensely diverse nature of heteropolysaccharides can be appreciated when it is realised that the Pneumococcus genus alone is divisible into at least 70 different types, each possessing a capsule which has a different chemical composition from the others, a difference registered by the immunological response of sera. The striking feature of bacterial hetero-polysaccharides, however, is the recurring 'theme' of a repeating unit, frequently of comparatively simple nature, a feature found

much less commonly in polysaccharides of the Plant kingdom. Indeed, it is possible that recent improvements in the methods used for investigating polysaccharides may result in a revision of the reported structures of some bacterial heteropolymers to somewhat simpler structures than have been claimed by earlier workers. From the vast range of polysaccharide investigations, a few pertinent examples have been selected.

(a) Aerobacter aerogenes A3 slime

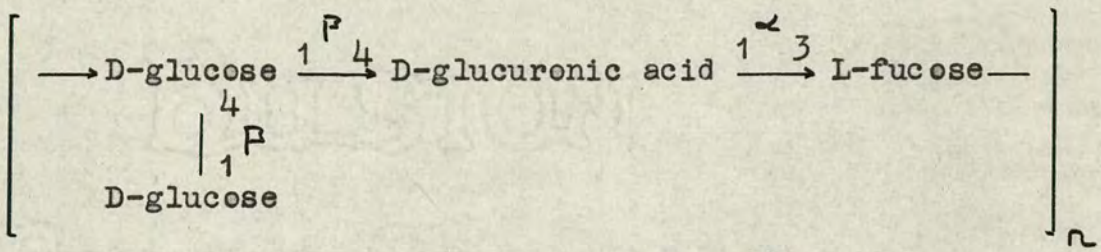
The related organisms Aerobacter, Escherichia and Klebsiella are of particular interest in this thesis since it is with regard to these genera that our investigations have been directed. Although the first encapsulated strains of E.coli were isolated by Smith et al in 1927, most research appears to have been concentrated on the somatic lipopolysaccharides rather than on extracellular material. The latter have been extensively catalogued serologically, but chemical investigation of the structures appears to have gone little further than the component monosaccharide level, sugars such as glucose, galactose, fucose, mannose and uronic acids figuring regularly. The structures of some of these polysaccharides will be described later.

With regard to the closely related organisms Klebsiella and Aerobacter, the methylation and partial-hydrolysis studies on the acidic slime polysaccharide of Aerobacter aerogenes A3 by Conrad et al (48,49) constitute a good example of intensive investigation of a particular bacterial slime or capsular polysaccharide. Particular care was taken to ensure complete methylation of the

polysaccharide, using a variation on the sodium-hydride - dimethyl sulphoxide method and comment is made on this method later in this thesis. Hydrolysis of the methylated, reduced, methylated polysaccharide (-OMe = 98% of theoretical) yielded equimolar amounts of 2,3,4,6-tetra-O-methyl-D-glucose, 2,3,6-tri-O-methyl-D-glucose (from 2,3-di-O-methyl glucuronic acid), 2,3-di-O-methyl-D-glucose and 2,4-di-O-methyl-D-glucose, analyses which were for a range of polysaccharides grown under a wide variety of growth conditions. The simplicity of the methylation results contrasts sharply with the complex picture derived by earlier workers (50) on the same polysaccharide, a complexity which must have resulted from partial demethylation of some of the sugar residues during hydrolysis or from an inherently undermethylated polysaccharide. Conrad and Sandford have shown, by elegant experiments, that demethylation occurred to the same extent with all the methylated sugars present, that it was minimal using the optimum hydrolysis conditions developed by Garegg and Lindberg, (51) and that partial demethylation was in any case always followed by further degradation to moities not recognisable as other methylated sugars such as to confuse the picture. On the basis of detection of free fucose in hydrolyses of methylated polysaccharide used by earlier workers, and on the basis of low yields of methylated sugars as a percentage of the original unmethylated polysaccharide, Conrad and Sandford conclude that undermethylation is a prime cause in the assumption that this and many other heteropolysaccharides are complex molecules. They suggest that standard techniques are subject to errors of considerable magnitude, and that structures of heteropolysaccharides may

be much simpler than has been realised.

Partial acid hydrolysis of the polysaccharide yielded a single neutral disaccharide, identified as cellobiose, and three glucuronic acid-containing oligosaccharides, a di-, a tri- and a tetra-saccharide -- each of which was found to have L-fucose at the reducing terminal. Sequence analysis, together with the methylation evidence indicated above, showed that the polysaccharide has the structure illustrated below:



Determination of the optimum partial hydrolysis conditions for accumlation of substantial quantities of each oligosaccharide was effected by measurement of the reducing value of hydrolsates by treatment of the end-groups with sodium ^{3}H borohydride and measuring on a scintillation counter the incorporation of ^{3}H into the mixture. Radiochromatographic techniques on these samples served to indicate the proportions of each oligosaccharide present after a given hydrolysis period, and by stopping the hydrolyses at different suitable times in separate experiments, each of the oligosaccharides mentioned above could be isolated in good yields. Since the positions of linkage in each sugar unit were known from the methylation results, it was only necessary to determine the sugar sequences and glycosidic configurations.

The aldobiuronic acid was identified by reduction of the methyl ester glycoside followed by hydrolysis to yield glucose and fucose. Periodate oxidation gave 1 mole of acetaldehyde per mole of aldobiuronic acid and confirmed the earlier methylation picture of the fucose units being substituted at position 3. Final confirmation was obtained by methylation of the aldobiuronic acid, followed by reduction and hydrolysis to yield 2,3,4 tri-O-methyl glucose and 2,4 di-O-methyl fucose, both of which were characterised.

The neutral disaccharide was converted quantitatively to glucose by β-glucosidase at a rate equal to that obtained with authentic cellobiose, the nature of the material being confirmed by chromatography and formation of a crystalline product.

The aldotriuronic and tetrauronic acids both yielded the glucuronosyl fucose on acid hydrolysis, yielding in addition one and two moles of glucose respectively. The tetrasaccharide yielded cellobiose as an intermediate in the hydrolysis, and it could be hydrolysed with β-glucosidase to yield glucose and the aldotriuronic acid. Fucose was confirmed as the end-group in the oligosaccharides by stoichiometric yields of acetaldehyde on periodate oxidation. The configuration of the glycosidic linkages in the oligosaccharides was determined by the use of specific enzymes and/or proton magnetic resonance spectroscopy. Periodate oxidation of the polysaccharide in buffered solution resulted in rapid consumption of 2 moles of periodate per tetrasaccharide unit corresponding to oxidation of the glucose end-group. Oxidation of

the main-chain glucose and glucuronic acid residues was much slower, completion of periodate consumption (2 further moles) only occurring after 300 hours. Fucose was found to be quantitatively intact.

Further evidence regarding the structure of the A3 slime has resulted from the use of bacteriophage enzyme (52). Sutherland has isolated two tetrasaccharide fragments, both having the basic structure indicated above but one having an acyl substituent, believed to be acetyl-; the addition of quantities of acetyl coenzyme A to the sugar-nucleotide mixture in a cell-free system containing the enzymes necessary for biosynthesis results in increased yields of the extracellular slime, and this together with other evidence suggests that the acetyl group is attached to a monomer or higher oligosaccharide prior to formation of the complete polysaccharide chain. At the time of writing, the position of acyl substitution is not known.

b) Other heteropolysaccharides

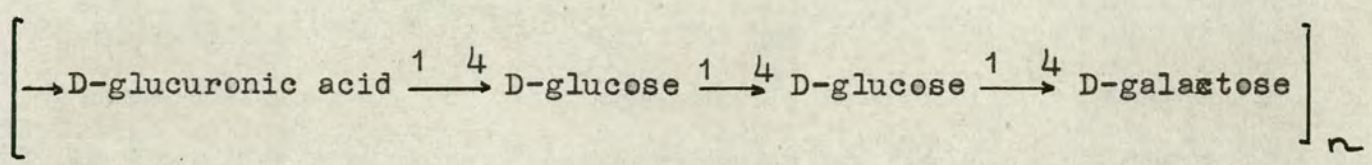
Of the wide range of polysaccharides which have received attention, those of the pneumococcus are comparatively well-identified. It should be stressed that while intensive chemical investigation has been employed in several instances, the results have been supplemented by the use of immunological cross-reactions, although the priority given to the latter technique might, perhaps, be considered excessive by a 'chemist'.

(i) Type VIII pneumococcus polysaccharide

As with many of the pneumococcus polysaccharides, preliminary information regarding structure was obtained by cross-reaction

of the material with an antiserum specific for another polysaccharide (Type III). The isolation of crystalline hepta-O-acetyl cellobiuronic acid as its methyl ester from both these polysaccharides (53) showed that the cross-reaction was attributable to the occurrence of multiple cellobiuronic acid units in these substances.

The fine structure of Type VIII polysaccharide has been determined by Jones and Perry (54). Quantitative analysis of the complete acid hydrolysate showed the presence of galactose, glucose and glucuronic acid in the ratio of 1:2:1, in agreement with the equivalent weight of the polysaccharide (703) which corresponds to a hexose polymer having one acid unit in four. A partial hydrolysate of the polysaccharide was fractionated to yield a series of oligosaccharides -- D-glucuronic acid, cellobiuronic acid, O-β-D-glucopyranosyl uronic acid-(1⟶4)-O-β-D-glucopyranosyl-(1⟶4)-D-glucopyranose, O-β-D-glucopyranosyluronic acid-(1⟶4)-O-β-D-glucopyranosyl-(1⟶4)-O-α-D-glucopyranosyl-(1⟶4)-D-galactopyranose and 4-O-(α-D-glucopyranosyl)-D-galactopyranose. Hydrolysis of the methylated polysaccharide and fractionation of the methylated sugars obtained afforded 2,3-di-O-methyl glucuronic acid (1 part), 2,3,6-tri-O-methyl glucose (2 parts) and 2,3,6 tri-O-methyl galactose (1 part). Reduction and methylation of the polysaccharide confirmed this methylation picture, and further evidence was the consumption of 1 mole of periodate per hexose unit, giving an overall picture of the polysaccharide structure as

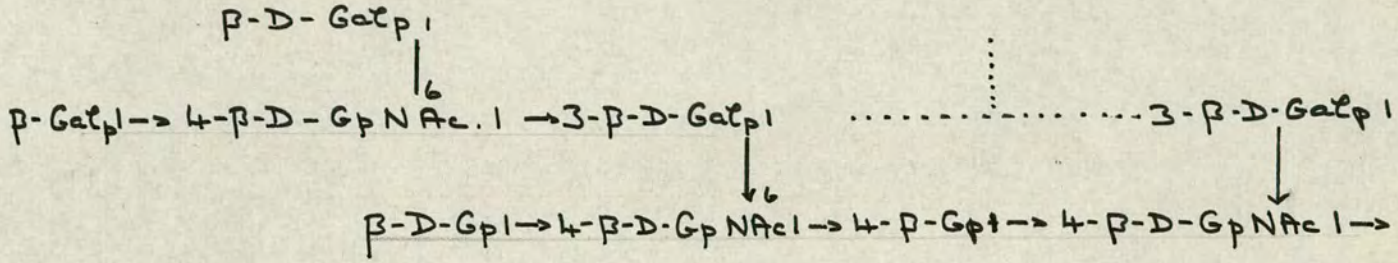


(ii) Type XIV pneumococcus polysaccharide

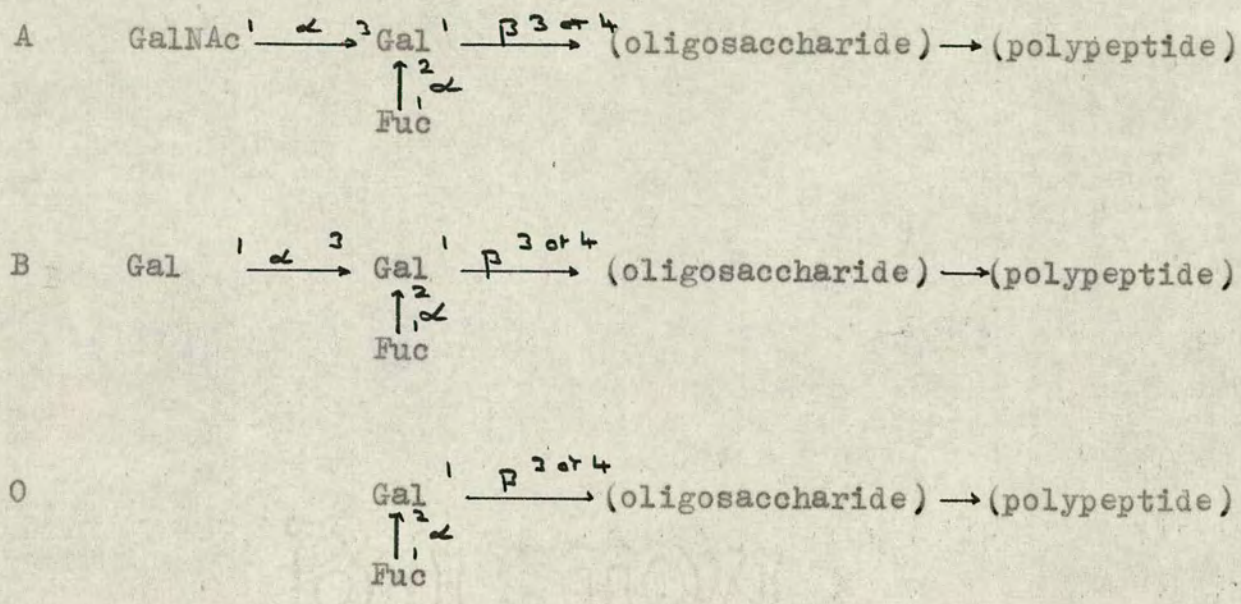
Type XIV polysaccharide is a classic example where immunological information was able to give substantial evidence as to some of the polysaccharide's structural features before any detailed chemical studies were initiated. To present the picture more coherently, however, the chemical evidence will be given first (55). Hydrolysis of the methylated polysaccharide (known to contain glucosamine, glucose and galactose) yielded 3-O-methyl glucosamine (1 part) and neutral sugars (2 parts) consisting of 2,3,4,6-tetra-O-methyl glucose (less than 4%) 2,3,4,6-tetra-O-methyl galactose and 2,3,6-tri-O-methyl glucose (69%) 2,4,6 tri-O-methyl galactose (23%) and traces of a dimethyl hexose. Thus 2 out of 6 galactose residues were non-reducing end-groups, galactose residues also being involved in 1:3 linkages.

Partial acid-hydrolysis of the polysaccharide gave, inter alia, two disaccharides which were assigned the structures 4-O-β-D-glucosyl-2-acetamido-2-deoxy-D-glucose and 3-O-β-(2-acetamido-2-deoxy-D-glucosyl)-D-galactose. All the linkages in the Type XIV polysaccharide were assigned the β-configuration on the basis of low specific rotation (+9.4°) and by the absence

of an infra-red absorption band at 840 cm^{-1}. The overall structure assigned to the polysaccharide is thus



While the ability of various plant and mammalian polysaccharides to cross-react with anti-Type XIV serum was important in indicating the likelihood of terminal galactose and 1,3 and 1,6 linked galactose residues being included in Type XIV polysaccharide, undoubtedly the most important galactose containing polysaccharides which cross-react with XIV serum are the blood group substances, A, B, AB and O all being agglutinated by the serum. It was found in 1948 (56) that heating at 100°C at a pH of about 2 destroyed the blood-group activity of the A, B and O substances but increased their capacity to react with Type XIV serum, a fact which can be readily explained with our present knowledge of the chemistry of the blood-group substances. The active portions of these substances bear a very close relationship to each other as can be seen below-



It is clearly apparent that mild acid hydrolysis is likely to remove fucose residues and/or N-acetyl groups, thus exposing galactose end-groups linked in the cases of A and B -1:3 to other galactose residues, the same terminal sequence as we apparently find in Type XIV polysaccharide.

Section I - Identification and estimation of component sugars in the slime polysaccharide of a K.12 E.coli

Part A

1. Preparation of extracellular slime polysaccharides
2. Purification of the polysaccharides
   - (a) attempted purification using phenol
   - (b) purification using chloroform-butanol

Part B

1. Identification of the component monosaccharides of the extracellular polysaccharides of S53.
   - (i) Small-scale investigation
   - (ii) Hydrolysis of polysaccharide and separation of products on ion-exchange and cellulose columns
     - (a) Separation of the neutral sugars
     - (b) Separation of the acidic sugars
2. Quantitative analysis of the component monosaccharides
   - (i) Semi-quantitative estimations
   - (ii) Quantitative analysis of neutral components
   - (iii) Quantitative estimation of glucuronic acid by decarboxylation
   - (iv) Analysis for ash content
   - (v) Analysis for nitrogen

Part C

Nature of glycosidic linkage in the aldobiuronic acid

- (i) Position of linkage
- (ii) Configuration of linkage

Part A

1. Mode of preparation and purification of extracellular slime polysaccharides.

Ten sterile trays (45 cm x 35 cm), with fitting lids, were filled with synthetic yeast-extract medium on agar support, the composition of the medium being,

Casamino Acids	1g.
Na_2HPO_4 (anhydrous)	10g.
KH_2PO_4	3g.
K_2SO_4	1g.
NaCl	1g.
$MgSO_4.7H_2O$	0.2g.
$CaCl_2$	0.01g.
$FeSO_4$	0.001g.
yeast extract	1g.
agar	20g.
glucose	2g.
H_2O	1 litre

It was subsequently found that an increase in the proportion of ferrous sulphate by a factor of 10 or 20 gave improved yields of slime, and later preparations were accordingly modified. The agar surface was covered with sterile cellophane and the trays were seeded with a fresh nutrient broth culture of E.coli K12, S53 or S61. After one week at 30°C, the resulting slime was scraped from the cellophane, residual traces being washed off the paper with dilute saline solution, and the resultant viscous

solution considerably diluted with saline/phosphate buffer. Bacterial cells were removed from earlier preparations of the slime by centrifugation at 15,000 G on a Spinco centrifuge, but it was subsequently found that the use of a Sharples continuous centrifuge was more than adequate for the somewhat undemanding requirements of the experiment. (The dry weight of intact cells and other debris was only of the order of 3% of the weight of the freeze-dried slime, a figure which was constant regardless of the method of cell-removal). The cell-free slime material was dialysed for four days against running water, and the resultant solution reduced in volume on a rotary evaporator to about 1.5 litres.

2. Deproteinisation of the slime

(a) Attempted removal of protein with phenol

To an approximately 2% solution of impure polysaccharide was added sufficient phenol to give a phenol concentration of 45%, and the solution was heated to 68°C at which temperature the phenol and aqueous phases merged. The mixture was left at 5°C in a cold room overnight whereupon the single phase had separated into a brown phenol/water layer and a turbid water/phenol layer, with an appreciable quantity of white suspension at the interface. The brown phenolic layer was withdrawn and the residue in the separating funnel re-treated with phenol in the manner described, a total of three phenol extractions being effected. After each successive treatment, the amount of interfacial suspension decreased noticeably, becoming almost nil after the final treatment.

The residual water/phenol layer was dialysed for 96 hours in running water, and the resulting solution reduced in volume on a rotary evaporator. Instead of the expected yield of approximately 10g of material, less than 500 mg was obtained.

The combined phenol extracts were treated with acetone to 80% and the precipitate taken up in water and dialysed against stationary water. No substantial phenol-sulphuric acid colouration could be detected in 1 ml aliquots of the water after a period of 24 hours, and the dialysis was continued against running water for a further 48 hours. The freeze-dried residue was found to be only weakly positive in the phenol-sulphuric acid test.

In a small-scale repeat experiment, the awueous layer was also examined by dialysis against stationary water, a very substantial increase in dialysable carbohydrate being apparent over 24 hours, as determined in the phenol-sulphuric acid test.

(b) Removal of protein by the butanol-chloroform (Sevag) method

Freeze-dried slime material (10g) was dissolved in water (1.5 L) containing sodium acetate (50g) and glacial acetic acid (25 ml), and the turbid solution shaken with chloroform (250 ml) and butanol (50 ml) for 30 minutes. On centrifugation, at 1500 rpm, a white, semi-solid emulsion separated between the aqueous layer and the chloroform. The turbid aqueous layer was decanted off and further treated with fresh chloroform-butanol solvent mixture, a total of seven such treatments being required before the phases showed no semi-solid emulsion. The combined chloroform/semi-solid emulsion layers were washed with water

(3 x 250 ml), and the combined aqueous washings further treated with chloroform-butanol.

The de-proteinised aqueous layers were dialysed for 4 days, reduced in volume on a rotary evaporator, and freeze-dried. Yield = 6g, mitrogen-content 0.5%.

Discussion

While the use of phenol is not as general as the use of butanol-chloroform, this is rather on account of its toxic nature than degradative properties that it might possess. It has been successfully employed in purification of antigenic substances from the typhoid bacillus (57), and although unsuccessful in removing final traces of protein from an E.coli K48 material, (58), no degradation was reported. The results of the phenol extraction of S53 and S61 slimes appear to indicate substantial degradation of the polysaccharide material, and the lability of the polysaccharides under these relatively mild conditions (pH 4.5, 65°C for 5 minutes) would indicate one or more very acid-labile linkages, possibly located in the 'core' of the polysaccharide rather than on the periphery.

The comparatively successful de-proteinisation using the mild butanol-chloroform method appears to confirm that glycoprotein linkages are not present in the extracellular slime elaborated by these organisms.

Part B

1. Identification of the component monosaccharides of the extracellular polysaccharide of E.coli K12, S53.

(i) Small-scale investigation

S53 polysaccharide, deproteinised as described in part A, (100 mg), was hydrolysed by heating on a boiling water-bath with sulphuric acid (2N, 20 ml) for 12 hours. The hydrolysate was neutralised with calcium carbonate, cooled in ice-water, filtered and concentrated on a rotary-film evaporator to a brown syrup containing traces of white salt. The concentrate was dissolved in water (1 ml) and examined by paper chromatography (solvents A and B, Spray no.1, paper, Whatman No. 1). In the case of both solvents, spots were obtained with identical R_F's to those of fucose, glucuronic acid, glucose and galactose, together with a faster-moving spot corresponding to glucurone, and a slow-moving spot to which was ascribed the general tentative structure of an aldobiuronic acid. The use of developing agents of more general reactivity such as periodate-benzidine (59) or silver nitrate/sodium hydroxide (60) did not reveal any further sugars in solvent systems A or B.

(ii) Hydrolysis of polysaccharide and the separation of products on ion-exchange and cellulose columns

Deproteinised S53 polysaccharide (2g) was hydrolysed, neutralised and concentrated as above. After dissolving in water (25 ml), the mixture of sugars was passed through a column of Amberlite IR 120(H^+)(bed-volume 100 ml, column diameter ¾"),

and the column washed with water until the eluate no longer gave a positive reaction to the phenol-sulphuric acid test (61). (2N hydrochloric acid was then passed through the column into a separate beaker, and the eluate tested for carbohydrate content in the same manner; no carbohydrate was detected.)

The sugar-containing eluate was reduced in volume to about 25 ml on a rotary-film evaporator and passed through Amberlite 400 (acetate) (bed-volume 100 ml., internal diameter ¾"), washing and testing being repeated as above. This eluate was reduced in volume to a syrup containing neutral sugars only, and elution of the resin column with N formic acid (500 ml) until negative to phenol-sulphuric acid, followed by evaporation to dryness on a rotary-film evaporator, gave a syrup consisting of acidic components. This latter syrup was left overnight in a vacuum desiccator over potassium hydroxide pellets.

(a) Separation and identification of the neutral sugars

A cellulose column (30 x 2.5 cm) was prepared by packing the cellulose in the form of an acetone slurry. The cellulose was finally conditioned by washing with acetone containing an increasing proportion of the final developing solvent, ethyl acetate/pyridine/water 16:4:1, until a solvent consisting solely of this solvent was passing through the column. The syrup containing neutral sugars was applied to the cellulose column, and the column eluted at approximately 30 ml per hour with the solvent indicated, 20 ml fractions being collected. The solution in every third tube was reduced in volume under reduced pressure and

the residue spotted onto a strip of filter paper and examined (spray A) for sugar content. Ranges of tubes found to contain sugar were reduced to syrups as soon as possible to minimise epimerisation and tar-formation in the solvent. The results of the chromatography are shown in Table 1.

Table 1 Chromatography of neutral sugars

Fraction	Tube no.	sugar present indicated by paper chromatography	yield
	1-80	none	25 mg
A	81-116	fucose	590 mg
	117-185	none	25 mg
B	186-222	glucose	300 mg
	223-254	none	12 mg
C	254-315	galactose	530 mg
	316-500	none	--

Characterisation of Fraction A

Fucose was characterised by the formation of the toluene-p-sulphonyl hydrazone (62). Formation of the derivative of authentic L-fucose was readily effected, but the presence of tarry solvent residues in fraction A appeared to contaminate the product. The accidental substitution of methylated sprits for methanol as a recrystallising medium resulted in a apparently uncontaminated derivative, but the melting point was substantially

less than that of the authentic material. Only after purification by preparative paper chromatography of the impure fraction A was derivative formation possible, crystals of m.pt. 170°C and mixed m.pt. 168-171°C being obtained. Fraction A was thus L-fucose.

Characterisation of Fraction B

Glucose was characterised by the formation of the 1,2:5,6-di-O-isopropylidene derivative (63). It was found in the course of making this derivative of authentic D-glucose, that shaking with the acetone-sulphuric acid mixture for periods substantially longer than the recommended 5 hours resulted in tar-formation, and 4 hours was found to be ample. The derivative of authentic D-glucose, after recrystallisation from ether/light petroleum B.pt. 40-60°C, had a m.pt. of 110°C. The derivative of fraction B had a m.pt. of 109°C and a mixed m.pt. of 109-110°C, thus confirming that fraction B was D-glucose.

Characterisation of Fraction C

Galactose was characterised by the formation of the N-methyl-N-phenylhydrazone (64). Using authentic D-galactose, white fluffy platelets of m.pt. 185°C were obtained, similar crystals but slightly brown being obtained from fraction C. These latter crystals, however, had an identical m.pt. and mixed m.pt. of 185°C, fraction C thus being confirmed as D-galactose.

(b) Separation of acidic components and identification of glucuronic acid

The solution containing the acidic fraction of the hydrolysate of S53 polysaccharide was chromatographed on a cellulose

column in the manner adopted for neutral sugars, but using an ethyl acetate/acetic acid /formic acid/ water 18:3:1:2 system to elute the sugars. The acidic mixture was resolved into three fractions as shown in Table 2.

Table 2 Chromatography of acidic sugars

Fraction	Tube no.	Sugar present indicated by paper chromatography	Yield
D	41-68	glucurone	140 mg
E	150-190	glucuronic acid	150 mg
F	300-345	aldobiuronic acid	84 mg

Characterisation of glucuronic acid and glucuronolactone

An attempt was made to characterise the glucuronic acid by formation of its 2,5-dichlorophenylhydrazone (65) but no recognisable derivative was obtained even when using authentic glucuronic acid. Accordingly, the glucuronic acid and the glucurone were both characterised as methyl β-D-glucofuranuronoside (6-3)lactone (66), a reaction which proceeded without difficulty.

m.pt. of authentic derivative 139.5

m.pt. of Fraction D " 139.5 mixed m.pt. 138

m.pt. of Fraction E " 138 mixed m.pt. 138

Thus fractions D and E would appear to be glucurone and D-glucuronic acid respectively.

2. Quantitative analysis of the component monosaccharides

(i) Semiquantitative estimations

In the course of isolating the component monosaccharides of S53 polysaccharide (Part B, 1), the yield of each component was noted. The results obtained have only a limited value since all the fractions contained appreciable quantities of tar-like contamination, but the results do afford a preliminary estimation of the proportions of each sugar present, and are shown in Table 3

Table 3 Approximate yields of component monosaccharides

Total weight of polysaccharide hydrolysed = 2g.

Fraction	Nature of sugar	Yield (mg.)	% of polysaccharide
E	aldobiuronic acid	84	---
(Thus, approximately 42 mg. of galactose and 42 mg. of glucuronic acid are involved in combined form in the aldobiuronic acid - Part C)			
A	fucose	590	29.5
B	glucose	300	15.0
C	galactose	530 + 42	28.6
D + E	glucuronic acid + glucurone	140 + 150 + 42	16.6
		Total % =	89.7

An attempt to perform a quantitative analysis by paper-

chromatography of a polysaccharide hydrolysate, followed by elution of fractions and determination by the phenol-sulphuric acid method, failed to give consistently concordant results, variations being apparent even in calibration systems, although in relative terms the approximate figures obtained were of a similar order to those obtained above.

(ii) Quantitative analysis of the neutral components by the aniline hydrogen phthalate method.

A quantitative analysis of neutral components of S53 polysaccharide was obtained using the aniline hydrogen phthalate dip method (67). A known weight of the polysaccharide was hydrolysed in 2N sulphuric acid for 15 hours at 100°C and neutralised with barium carbonate. After cooling in ice-water, filtering and making up to a known volume in a standard flack, aliquots were chromatographed in solvents and, standards for calibration being run on the same paper. The papers were dipped in the aniline hydrogen phthalate reagent and the sugar-spots developed by heating for ten minutes in a 105°C oven. The coloured spots were eluted using a 3 ml. portion of the ethanolic hydrogen chloride solution, and the solutions transferred to tubes for reading in an SP 500 spectrophotometer. From the readings obtained with the standard solutions, calibration curves were obtained for each sugar, and the sugar contents in the hydrolysates determined from these. Results were found to be very reproducible, and those shown below were sample figures obtained from one analysis.

Results

Weight of polysaccharide taken	0.0875g
Volume of neutral hydrolysate	10 ml.
Volume of sample for chromatography	0.025 ml.
Volume of eluting solvent	3 ml.

Sugar	colorimeter reading of phthalate complex	µg from calibration chart
fucose	0.400	50.0
glucose	0.175	45.3
galactose	0.222	60.7
aldobiuronic acid	0.81	16.0

Calculations

Fucose 50 µg in 25 µl $\frac{50/25 \times 1/100}{0.0875} \times 100\%$

= 22.8% fucose

Similarly,

glucose content = 20.7%

(free) galactose content = 27.7%

aldobiuronic acid = 7.3%

(Thus combined galactose constitutes 180/356 x 7.3 = 3.66%)

Conversion of these figures to the percentages as anhydrides gives the following results:-

fucose	20.3%
glucose	18.6%
galactose (total)	28.19%

(iii) Quantitative estimation of glucuronic acid by decarboxylation

Glucuronic acid analysis was kindly effected by Dr. G.M. Cree using the decarboxylation technique of Anderson (68). Reproducible

figures of 18.3% (18.29 and 18.31 %) were obtained.

(iv) Analysis for ash-content

A crucible and lid were heated to constant weight on a Meker burner. A sample of polysaccharide was ignited in an identical manner and the residual ash determined by increase in weight of the crucible. The results are given below:

weight of crucible and lid	=	21.0177g
weight of crucible and lid + polysaccharide	=	21.0795g
weight of crucible and lid + ash	=	21.0220g
weight of ash	=	0.0043g
and weight of polysaccharide	=	0.0618g

Thus percentage ash = 6.95%

Other samples yielded percentage ash values of 10.8, 5.8 and 8.2 respectively. Polysaccharide in the H^+ form yielded very similar figures of 6.1 and 8.8, while S53 lipopolysaccharide gave slightly lower values of 4.4 and 5.1.

(v) Analysis for nitrogen

Nitrogen analysis using a micro-Kelhdal technique was kindly performed by Dr. G.M. Cree. A result of 0.5% was obtained, although subsequent samples (kindly analysed by Miss Lorna Tait) frequently showed higher figures of 0.8 - 1.2%, and no sample gave a figure as low as 0.5%.

Summary of results and discussion

The percentage constitution of sugars, and the nitrogen and ash-content analyses, were

fucose	(as anhydride)	20.3
glucose	(" ")	18.6
galactose	(" ")	28.2
glucuronic acid	(" ")	18.3
protein (estimated as 6.3 x nitrogen content)		3.2
ash content		6.95

The combined sugar and protein percentages total only 88.6, and while the proportion of metal ions originally present in the native polysaccharide cannot be accurately estimated from the ash content figure, it would appear that approximately 10% of the polysaccharide cannot be accounted for. Evidence will be produced in later chapters of this thesis for the presence of an acid-labile constituent in the polysaccharide. Evidence will also be produced to show that the difficulties encountered in obtaining a crystalline derivative of L-fucose in polysaccharide hydrolysates may have stemmed from contamination of the sugar by a breakdown product of this unidentified component.

Part C

Nature of glycosidic linkage in the aldobiuronic acid.

(i) Position of linkage

Extended acid hydrolysis of the aldobiuronic acid and chromatography of the products showed that the components were glucuronic acid and galactose, together with smaller quantities of flucurone and unchanged material. The degree of polymerisation, as determined by the phenol - sulphuric acid method on the material (69) and borohydride-reduced material, implied that the compound was a disaccharide (actual value = 2.2) of general structure G.Acid $\xrightarrow{1\ 3}$ gal.

To determine the position of linkage in the galactose ring, the aldobiuronic acid was methylated and reduced, and then remethylated to give a fully methylated, reduced derivative.

Aldobiuronic acid (90mg) was dissolved in cold 10% sodium bicarbonate solution (50ml), and the solution stirred under an atmosphere of nitrogen in an ice-bath for one hour. 30% sodium hydroxide (30 ml) and dimethyl sulphate (10 ml) were added dropwise over a period of 3 days, the solution being maintained at 0-5°C during the first 2 hours. The solution was acidified to pH 2 with 6N sulphuric acid and extracted with chloroform (3 x 50 ml); the chloroform extracts were combined, washed with water, dried over anhydrous sodium sulphate, filtered and evaporated to dryness on a rotary film evaporator. The partially methylated material (85 mg) was dissolved in dry dimethyl formamide (10 ml) containing methyl iodide (1 ml) and was cooled in ice-water. To the solution was added silver oxide (500 mg) the solution then being shaken for 1 hour at 0-5°C. and thereafter at room temperature for 3 days. The solution was taken to dryness on a rotary film evaporator at 100°C, chloroform (20 ml) was added, and the suspension allowed to stand for 2 hours. The suspension was filtered through glass paper and taken to dryness on a rotary film evaporator to yield 70 mg of a brownish residue

(a) Gas-liquid chromatographic investigation

The product (5 mg) was methanolysed (see general methods) and examined on a Pye 104 Gas chromatograph with a hydrogen flame detector and using column no. 1 . The sample was also examined on a Pye Argon chromatograph using column no. 2 . The retention times obtained are shown in Table 4.

Table 4

Retention times of components of methanolysed, methylated aldobiuronic acid.

sugar standards	retention times column 1	retention times column 2
2,3,4,6 Me^4 glucose	1.00 1.43	1.00 1.38
2,4,6 Me^3 galactose	3.13m 3.54s	4.11m 4.69s
2,3,6 Me^3 galactose	2.52s 3.18w 3.50m	3.11s 3.72w 4.11w 4.50m
3,4,6 Me^3 galactose	4.11	4.99
reduced, methylated aldobiuronic acid	1.00 1.42	1.00 1.38
	3.14 3.53	4.09 4.69

Thus the methanolysate would appear to contain only 2,3,4,6 tetra-O-methyl glucose and 2,4,6 tri-O-methyl galactose.

(b) Paper chromatographic investigation

The methylated, reduced, methylated aldobiuronic acid was hydrolysed in formic acid (45% at 100°C for 10 hours), the formic acid was removed, and the resulting syrup left overnight in a vacuum oven in the presence of potassium hydroxide pellets. Chromatography on 3MM Whatman paper using solvent system no. G, was followed by elution of the two fractions obtained using methanol, and the resulting eluates taken to dryness on a rotary film evaporator.

The sugars were found to have R_F corresponding to tetra-O-methyl glucose and 2,4,6 tri-O-methyl galactose in solvents F and G.

The tri-O-methyl galactose was unambiguously identified by recrystallisation from a chloroform/petrol ether mixture, impurities tending to be precipitated first. Small white crystals of methylated sugar were collected dried and identified.

authentic 2,4,6 tri-O-methyl galactose	m.pt.	84°
tri-O-methyl galactose from hydrolysis	m.pt.	84°
mixed	m.pt.	85°

No derivative of the tetra-O-methyl glucose was prepared, and its identity was assumed on the basis of its chromatographic mobility and the earlier identification of glucuronic acid as being the acidic component of the disaccharide.

(ii) Configuration of the linkage

The specific rotation $[\alpha]_D$ of the disaccharide was determined to be +38° at a concentration of approximately 2%, and by comparison with the rotations of analogous disaccharides listed in Table 5, this would appear to be indicative of β-linkage rather than an α-linkage. To eliminate the contributions to the rotation figure by the anomers about C_1 of the reducing end of the disaccharide, the material was reduced overnight with sodium borohydride, excess borohydride removed with acetic acid, the solution passed through a short column of cation-exchange-resin IR 120 (H^+), and the boric acid contaminant removed by codistillation with methanol under reduced pressure. A specific optical rotation $[\alpha]_D$ of +48 was obtained.

Table 5 Specific rotations of some disaccharides containing glucuronic acid. (70)

4-O-α-D-glucopyranosyluronicacid-D-galactose	+110
4-O-β-D-glucopyranosyluronicacid-D-galactose	+15
6-O-β-D-glucopyranosyluronicacid-D-galactose	-3
4-O-β-D-glucopyranosyluronicacid-D-glucose	+7.6

While the specific rotations of aldobiuronic acid and aldobiuronitol were perhaps more indicative of a β-linkage, a commercial β-glucuronidase did not appear to act on the disaccharide (personal communication by Dr. I.W. Sutherland, Dept. of Bacteriology). The disaccharide was accordingly examined by proton magnetic resonance spectroscopy in D_2O solution (Perkin-Elmer 60 m/cs), having previously been deuterated by freeze-drying three-times in D_2O. Among the peaks was a doublet at approximately 4.77, but since the contribution from the proton on the anomeric carbon atom of the galactose residue might have been responsible for this, the aldobiuronitol was also examined in the deuterated form. Table 6 shows the positions of glycosidic proton resonance for standard disaccharides where the glycosidic linkage is α- ($H_G\alpha$) or β- ($H_G\beta$). The figure in brackets is the coupling constant. (Reference 71).

Table 6

	$H_G\beta$	$H_G\alpha$
glucose $\xrightarrow{1\beta 4}$ glucose	5.50 (7.0)	
glucose $\xrightarrow{1\beta 6}$ glucose	5.50 (7.0)	
glucose $\xrightarrow{1\alpha 1}$ fructose		4.59 (3.2)
glucuronic acid $\xrightarrow{1\alpha 2}$ fructose		4.70 (3.2)
galacturonic acid $\xrightarrow{1\beta 4}$ glucose	5.58 (7.1)	
galacturonic acid $\xrightarrow{1\alpha 6}$ glucose		5.04 (2.9)

aldobiuronitol gives doublet at 4.87 (coupling constant approx. 3)

From these figures, it would appear that the glycosidic linkage in the aldobiuronitol, and hence in the aldobiuronic acid, is α .

Note Roden and Markovitz (72) have assigned a β-linkage to this aldobiuronic acid on the basis of cleavage by a liver β-glucuronidase; the exact source of the enzyme is not indicated in their paper, but this result would appear to be open to review in view of the evidence listed above for an α-linkage.

Section 2 Methylation of S53 polysaccharide; hydrolysis and identification of products

1. Attempted methylation using sodium hydroxide and dimethyl sulphate.

2. Methylation using the same reagents in aqueous sodium bicarbonate.

3. Methylation in aprotic solvents

   (a) Esterification of the polysaccharide

   (b) (i) Methylation using potassium hydroxide and methyl iodide

   (ii) Barium hydroxide methylation in N-methyl-2-pyrrolidone

   (iii) Barium hydroxide methylation in hexamethyl phosphoramide

4. Complete methylation of the polysaccharide using silver oxide and methyl iodide

5. Hydrolysis of the methylated polysaccharide, separation and identification of component sugars.

6. Gas-liquid chromatography of component methylated sugars

7. Summary of the methylation results and discussion of the methylation methods attempted.

1. Attempted methylation using sodium hydroxide and dimethyl sulphate

Purified polysaccharide (5g) was dissolved by stirring in water (550 ml.). A stream of nitrogen was passed through the stirred solution for one hour, the flow of nitrogen being continued while sodium hydroxide solution (30%, 120 ml.) and dimethyl sulphate (40 ml.) were added at 0°C over three hours. Further additions of these reagents were made at 24 hour periods at room temperature for one week, after which time the volume of the reaction mixture became inconveniently large. The solution was dialysed for 4 days and freeze-dried to give a yield of 3g. of material (-OMe = 16.8%).

The partially methylated material was subjected to two further methylation cycles as above, a total of 18 additions of reagents having thus been effected. The resultant dialysed, freeze-dried material (3g.) was found to have a low methoxyl content (4.5%) and a weak phenol sulphuric acid response, nor were any sugars apparent after treatment of the residue with 45% formic acid at 100°C for 4 hours. The low yield of material was obviously unrepresentative of 5g of starting material, and it was thus apparent that the above methylation conditions were not suitable for methylation of S53 polysaccharide. A possible reason for the failure of the experiment is suggested in the methylation summary (Section 2 Part 7).

2. Methylation with sodium hydroxide and dimethyl sulphate in aqueous sodium bicarbonate

S53 polysaccharide (200 mg) was dissolved in 10% sodium bicarbonate solution and methylated as above. The dialysed, freeze-dried product gave a strong phenol sulphuric acid response, and had a methoxyl content of 19.5%. A portion of this partially methylated material was treated with methanolic hydrogen chloride (3%, 2 ml) at 100° for 16 hours, and was worked up in the manner described in 'general methods', page GM1, the resulting methanolysate being examined on a Pye Argon chromatograph on column no 2 (page GM3) at 175°C. The resulting overall picture was very similar to that obtained with subsequent samples of methylated polysaccharide as described in part 5.

3. Methylations in aprotic solvents

Aprotic solvents such as dimethyl formamide, dimethyl sulphoxide and N-methyl-2-pyrrolidone have considerable application in the methylation of soluble polysaccharides. Certain characteristics of these solvents, coupled with the absence of water (which competes for the reagents in the method described in part 1), make methylation a much more efficient process. S53 polysaccharide is insoluble in such solvents unless the ionic carboxylic acid salts are converted to non-ionic organic esters.

(a) Esterification of S53 polysaccharide (73)

S53 polysaccharide (5g) was dissolved in water (3l) and was converted to the acid form by passing the solution through a

column of Amberlite IR 120 (H^+) ion-exchange resin of bed-volume 1.5 litres. The volume of the solution was reduced to about 1 litre, and a solution of 50 ml. ethylene oxide in 100 ml. of water added after first cooling the polysaccharide solution to about 5°C. The mixture was then securely stoppered and left at room-temperature for 28 days; during this period, the pH of the solution rose from 3.3 to 4.8, the latter being the pH of the distilled water available in the laboratory, and it was inferred that ethylene oxide does not react with carbonic acid such that the pH is raised above 4.8.

The solution was extensively dialysed, the chief purpose of this being to remove a contaminating by-product, the presence of which makes re-dissolution difficult after the polysaccharide has been freeze-dried. The yield of freeze-dried material was 4.6g. (It was subsequently found that the use of propylene oxide instead of ethylene oxide gave faster esterification.)

(b) (i) Methylation using potassium hydroxide and dimethyl sulphate (74)

Esterified S53 polysaccharide (700 mg) was dissolved with warming in dimethyl sulphoxide (100 ml), dimethyl sulphate (3 ml) was added and the mixture cooled to 0°C. To the stirred solution, through which a steady stream of nitrogen was passed, potassium hydroxide pellets (10g) were added in 0.5g portions over 8 hours. The mixture was stirred a further 24 hours and filtered, and the filtrate extracted overnight with chloroform in a continuous liquid-liquid extracter. The chloroform was

washed with water (3 x 100 ml), dried over anhydrous sodium sulphate and evaporated to dryness on a rotary-film evaporator; yield = 100 mg. Although this yield was appreciably better than the one obtained using the sodium hydroxide dimethyl sulphate method described in part 1, it was still too small to be considered representative of the 700 mg of starting material. In view of this and the poor yields obtained by another worker in the department (Dr. S.E.B. Gould) in methylating pectic material by this method, further investigation along these lines was discontinued.

(ii) Barium hydroxide methylation in N-methyl-2-pyrrolidone

Esterified polysaccharide (2.3g) was dissolved in N-methyl-2-pyrrolidone (200 ml) with warming. The solution was cooled and methyl iodide (10 ml), dissolved in N-methyl-2-pyrrolidone (20 ml), was added with stirring, the solution then being cooled to 0°C. Barium hydroxide octahydrate (20g) was added to the cold solution and the mixture shaken for 2 hours at 0°C and for 20 hours at room temperature. Reagents were added and shaking continued for two further 24-hour periods.

The extraction of methylated polysaccharide using chloroform proved to be impracticable on account of the formation of stable emulsions, but the material was easily and conveniently isolated via dialysis over 3 days and subsequent freeze-drying. The yield of almost white, freeze-dried material was 2.0g (-OMe = 27.3%).

(iii) Barium hydroxide methylation in hexamethyl phosphoramide

The use of hexamethyl phosphoramide as a solvent for nucleophilic reactions in organic chemistry led to an attempt to methylated the polysaccharide in this solvent using the same reagents as in part 3b (ii) above. Greater difficulty was encountered in dissolving the polysaccharide ester than was the case with N-methyl-2-pyrrolidone, but the experimental details were analogous to those used above. From 200mg of ester, a yield of 175mg of methylated material (-OMe = 13%) was obtained.

4. Complete methylation of the polysaccharide using silver oxide and methyl iodide

Partially methylated polysaccharide (2g) (prepared using the barium hydroxide-methyl iodide method with N-methyl-2-pyrrolidone as solvent) was dissolved in dimethyl formamide (40 ml) in a 100 ml conical flask. To the solution was added dry, finely-powdered silver oxide (6g) and methyl iodide (5ml.), a double-surface water condenser equipped with calcium chloride drying tube was attached to the flask, and the solution was stirred magnetically at 35°C for two days. The cooled mixture was diluted with chloroform and filtered. The filtrate was washed quickly with 1% sodium cyanide solution to remove silver salts, the cyanide solution then being back-washed with chloroform (3 x 100 ml), these latter chloroform extracts being combined with the original chloroform solution. The chloroform solution was washed with water (3 x 100 ml) and dried over anhydrous sodium sulphate. The methlyated

polysaccharide was precipitated from chloroform solution with light petroleum (60 - 80° fraction) and dried in a vacuum oven at 60° overnight. Yield = 1.6g -OMe = 33.8%.

The methylation was repeated a further three times, after which treatment the yield of recovered material had been reduced to 1.15g and the methoxyl content had risen to only 35.8%. Reports from other workers that the particular batch of commercial silver oxide was less effective than usual led to the methylation being repeated with fresh material and fresh Ag_2O. In addition, all equipment and reagents were carefully dried. Possible de-esterification during the work-up was avoided by not employing sodium cyanide to remove silver salts, the solution instead being diluted 4 times with chloroform and left to stand overnight; the filtered solution was evaporated to dryness on a rotary film evaporator using toluene to remove dimethyl formamide by codistillation, and the residue dissolved in chloroform, extracted with water as above, and precipitated from the sodium sulphate dried solution with petrol ether. Employing these modifications and precautions, the methylation was continued a further 4 times, but the methoxyl value appeared to be stationary at approximately 38.5% and the overall yield was 0.81% from 2.3g of esterified native polysaccharide.

Hydrolysis of native polysaccharide reveals an appreciable colloidal content which may be inorganic, and the possibility of contamination of the methylated polysaccharide by such material was investigated by analysing the product for ash content.

A value of less than 0.1% was obtained, and thus the low methoxyl value was not due to ash contamination. The possibility of organic impurities was investigated by fractional precipitation of the methylated polysaccharide from chloroform solution using light petroleum (40 - 60° fraction). The precipitated fractions were dried in a vacuum oven overnight at 60°C and then analysed for their methoxyl content. The results of the analyses are shown in Table 7.

Table 7 Methoxyl contents of precipitated fractions of methylated polysaccharide

percentage concentration of petroleum ether	relative amounts of material precipitated	-OMe%
up to 50% m	none	
66	+	32.0
72	+++	34.1
75	+++	33.7
78	++++	36.9
80	+++++	39.0
85	+	18.9
over 90	insufficient to analyse	

Examination of each fraction by methanolysis and gas-liquid chromatography revealed no apparent differences in the pattern of methylated sugars present.

Any polysaccharide consisting of only hexose units has the general formula $H-(C_6H_{10}O_5)_n-OCH_3$, and it follows that for high molecular weights (n greater than 300), the calculated theoretical methoxyl value is 45.6) If we assume that every uronic acid carboxyl group is esterified, and that our unidentified monosaccharide component has a hexose structure (for lack of other information at this stage), then the theoretical methoxyl content is reduced by the presence of 20.3% of fucose (as anhydride units) - i.e. 1/5 of the methylated polysaccharide has 2 methoxyl units instead of 3, giving an approximate theoretical methoxyl content of 42 - 43%.

To be certain of complete esterification of the uronic acid carboxyls, a chloroform solution of the polysaccharide was passed through a column of Amberlyst 15 ion-exchange resin (bed volume 50 ml), the solution then being treated with ethereal diazomethane. After removal of excess diazomethane, the treatment was repeated, and the fully esterified methylated polysaccharide isolated as a light petroleum precipitate and dried in a vacuum oven overnight. Examination of the infra-red spectra before and after treatment with diazomethane showed a pronounced decrease in the proportions of a peak at 1665 cm^{-1} which can be attributed to the carboxylate ion CO_2^- (75). In addition, a pronounced hydroxyl peak at 3600 cm^{-1} was diminished to very small proportions, although not completely eliminated. The -OMe content of this fully esterified material was found to be 40.8%

A further treatment of this esterified material with

silver oxide and methyl iodide, followed by treatment with Amberlyst 15 and diazomethane did not improve the methoxyl value, a figure of 40.8% appearing to be the limit obtainable using the reagents and techniques available, and it was accordingly on this material that all subsequent experiments were performed.

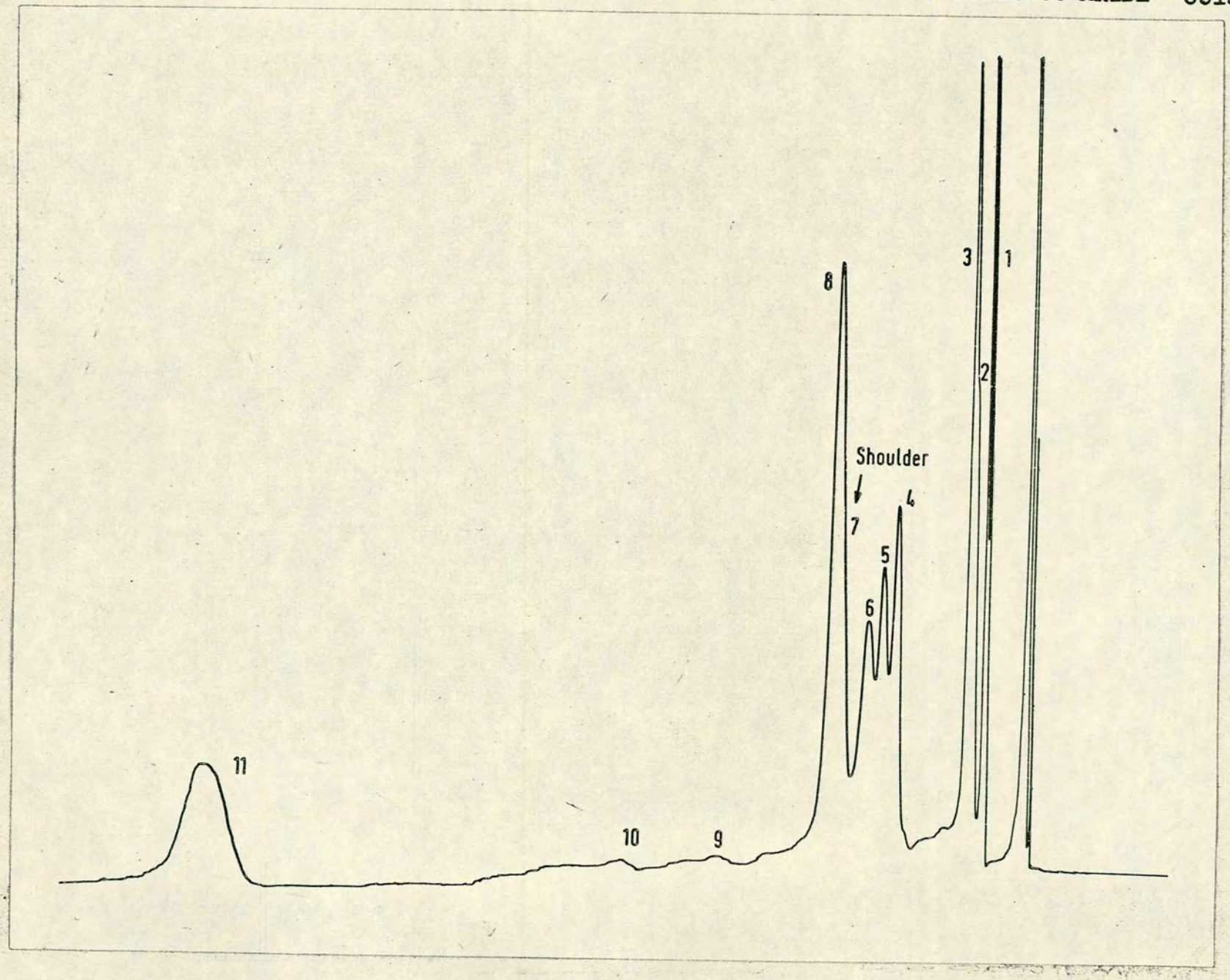
5. Hydrolysis of the methylated polysaccharide; separation and identification of component sugars

Reference has been made in Section 1 to the possible presence of an acid-labile component monosaccharide in the S53 extracellular polysaccharide; it was as a result of the work described in this part of Section 2 that more concrete evidence was obtained.

(a) Preliminary investigation

Methylated S53 polysaccharide (0.2g) was hydrolysed in 45% formic acid for 18 hours at 100°C, the solution was taken to dryness on a rotary-film evaporator, and the residual syrup left overnight in a vacuum oven in the presence of potassium hydroxide pellets. By the use of preparative paper chromatography (Whatman 3mm) in a variety of solvents, many of the components were separated as pure fractions which could be identified tentatively either by chromatographic mobility or by methanolysis and examination of the products on a gas-liquid chromatograph. A more systematic and quantitative analysis will be described in succeeding paragraphs. Of particular interest, however, was a sugar which had very high R_f values in many solvents, in particular in benzene:ethanol:water

FIGURE 1. GAS CHROMATOGRAM OF A METHANOLYSATE OF S53 METHYLATED POLYSACCHARIDE Column no.1

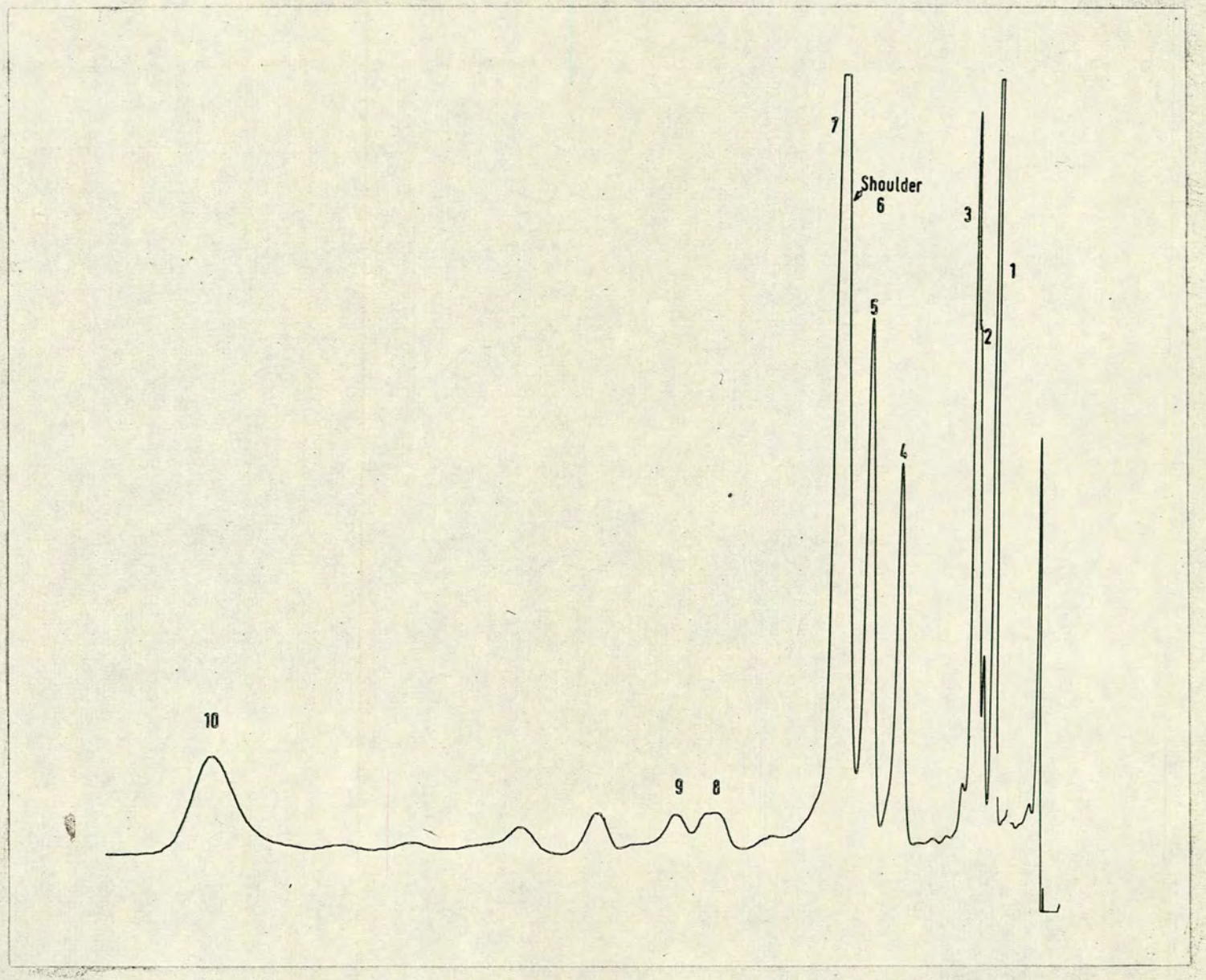


169/47/15, in which solvent it was faster than tetra-O-methyl glucose standard. Methanolysis and examination using gas chromatography on columns 1 and 2 showed that it had an astonishing retention time of the order of 15 as compared with typical dimethyl sugars having retention times of approximately 8. (A typical gas-liquid chromatogram of methanolysed, methylated S53 polysaccharide including the unidentified component (hereinafter termed 'X') is shown in Fig. 1) While extended methanolysis appeared to have little effect on the sugar glycoside, extended treatment of the sugar with 45% formic acid (30 hours at 100°) resulted in considerable reduction in spot intensity after chromatography and spraying with p-anisidine hydrochloride. Since a whole Section (6) is devoted to this methylated sugar, further comment will be reserved until appropriate, although the partial lability of this moiety to acid had regrettable consequences in the full investigation of a hydrolysate of the methylated polysaccharide as described in part 5 (b).

(b) Full investigation of the components

Methylated polysaccharide (1.5g) was hydrolysed in formic acid (45%) for 24 hours at 100°C, and the hydrolysate was worked up in the manner described in the preliminary investigation. The syrupy hydrolysate was applied to a cellulose column (length 28 cm, internal diameter 5 cm) equilibrated against a solvent consisting of light petroleum (100-120 fraction) and butanol in the ratio of 3 to 1, and the column was eluted with the same solvent, 25 ml fractions being collected. A chromatographically homogeneous

FIGURE 2. GAS CHROMATOGRAM OF A METHANOLYSATE OF METHYLATED S53 POLYSACCHARIDE Column no.2.



fraction (I) was obtained. Continuing the elution using the same mixture of solvents but in a 1:1 ratio, three further fractions (II, III and IV) were obtained, none of which was chromatographically homogeneous. Using a 1:3 mixture of the same solvents, a chromatographically homogeneous fraction (V) was obtained. The column was finally eluted with butanol half-saturated with water to give two further fractions (VI and VII) which were again chromatographically homogeneous.

Fraction I

By virtue of its chromatographic mobility, this fraction was presumed to be the unidentified sugar X previously detected in the preliminary investigation, and methanolysis and examination by gas-liquid chromatography (see later table) confirmed this. The yield was 25 mg -- very small compared with that obtained in the preliminary investigation.

Fraction II

This fraction (125 mg) was found to be almost homogeneous in a variety of chromatographic solvents, and the major component was obtained by chromatography on 3mm Whatman paper in butanol/ethanol/water 4:1:5, the traces of minor component running appreciably faster than the brown spot obtained after spraying a sample paper with p-anisidine followed by development in a 100° oven. The eluted major component had paper- and gas-liquid-chromatographic mobilities, (Table 8) indicative of 2,3-di-O-methyl fucose, and the identity was confirmed by formation of the methyl-α-pyranoside (76) which was recrystallised from light petroleum.

melting point of derivative	48° - 50°
literature melting point	49° - 51°

Fraction III and Fraction IV

Fractions III and IV were found to be mixtures of the same two sugars (as indicated by chromatography in several solvents), one of which appeared to be the same sugar as the minor component of Fraction II. Separation and purification was effected on a small cellulose column using a solvent of methyl ethyl ketone/water/ammonia 400:34:1. By paper chromatography in several solvents, and by gas-liquid chromatography (Table 8), the two components were tentatively identified as 2,4,6 tri-O-methyl glucose (275 mg) and 2,4,6 tri-O-methyl galactose (420 mg), these identities being confirmed by formation of crystals.

2,4,6 tri-O-methyl glucose was identified as its α-anomer by repeated recrystallisation from ether (77) as platelets.

M.pt. of column fraction	120°
M.pt of authentic material (isolated from methylated laminarin)	122°
Mixed m.pt.	120°

2,4,6 tri-O-methyl galactose was crystallised from ether/light petroleum to yield small needles;

M.pt. of column fraction	100°
M.pt of authentic material	98° - 101°
Mixed m.pt.	100°

Fraction V

The homogeneous fraction V was tentatively identified by gas-liquid chromatography (Table 8) as 2-O-methyl fucose, this being confirmed by direct crystallisation which occurred readily of the sugar syrup, a yield of 140 mg being obtained.

M.pt of crystalline sugar	147 - 150°
Literature m.pt. (78)	147 - 149°

Fraction VI

The minor fraction (23mg) had paper-chromatographic mobilities similar to those of di-O-methyl hexoses, and complete methylation of a sample using silver oxide and methyl iodide, followed by examination on a gas-liquid chromatograph, showed tetra-O-methyl glucose, the original sample thus being a di-O-methyl glucose. Chromatographically distinguishable from 2,4-di-O-methyl glucose and 4,6-di-O-methyl glucose, the material was found to be periodate-labile as its methyl glycoside; since the methyl glycosides of all the other dimethyl glucoses would be stable to periodate the minor component was provisionally assigned the structure of 2,6-di-O-methyl glucose. In view of the trace quantities of this material, no further characterisation was attempted.

Fraction VII

Fraction VII (116mg) was chromatographically homogeneous in a number of solvent systems and was electrophoretically mobile in pyridine acetic acid buffer. Gas-liquid chromatography indicated that it was 2,3-di-O-methyl glucuronic acid, this being confirmed by formation of the methyl ester methyl glycoside-4-p-nitrobenzoate (79). The ester-glycoside was prepared using 3% methanolic hydrogen chloride in the usual manner and the product was dissolved in the minimum quantity of pyridine to which was added p-nitro-benzoyl chloride (100mg.). After 4 days, the residue was triturated with a saturated solution of sodium bicarbonate and the discoloured precipitate filtered off. Four

recrystallisations from ethanol were necessary to give small white platelets (60 mg).

M.pt. of derivative 155^{o}

Literature m.pt. 157^{o}

6. Gas-liquid chromatography of the component methylated sugars

Each of the seven impure fractions obtained from hydrolysis of methylated S53 polysaccharide was methanolysed and examined by gas-liquid chromatography. The pure fractions were similarly examined. No peaks were obtained on the chromatograms of the impure samples which could not be accounted for on chromatograms of the pure fractions, and this was accepted as indicating that no component was present other than those listed in part 5 above. The results of gas-liquid chromatography of these pure fractions and of reference samples are listed in Table 8.

Table 8 Gas-liquid chromatography of standard sugar glycosides and fractions from hydrolysis of methylated S53 polysaccharide.

sugar glycosides (reference samples)	columns (i)		columns (ii)	
2,3,4,6-tetra-O-methyl glucose	1.00m	1.38s	1.00m	1.43s
2,4,6-tri-O-methyl glucose	2.56m	3.64s	3.28m	4.82s
2,3,6-tri-O-methyl glucose	2.78m	3.64s	3.56m	4.81s
2,3,4-tri-O-methyl glucose				
2,4-di-O-methyl glucose	5.88m	7.85s	10.1m	13.7s

Table 8 (cont.) Gas-liquid chromatography of standard sugar glycosides and fractions from hydrolysis of methylated S53 polysaccharide.

sugar glycosides (reference samples)	columns (i)	(ii)
4,6-di-O-methyl glucose	5.92s 7.30m	
2,3-di-O-methyl glucose	7.2m 9.20s	
2,3,4,6-tetra-O-methyl galactose	1.70	1.89
2,4,6-tri-O-methyl galactose	3.08m 3.50s	4.08m 4.69s
2,3,6-tri-O-methyl galactose	2.52s 3.18w 3.50m	3.11s 3.72w 4.11w 4.50m
2,3-di-O-methyl galactose	6.56s 9.80m	
2,6-di-O-methyl galactose	5.21w 6.08s 6.98w 7.77m	
4,6-di-O-methyl galactose	5.15s 8.40m	8.28s 14.6m
2,4-di-O-methyl galactose	9.51m 10.70s	8.35m 8.93w 10.6vw 11.43s
2,3,4-tri-O-methyl fucose	0.72	0.85
2,3-di-O-methyl fucose	0.9s 1.15sh 1.18m 1.24w	1.05s 1.44sh 1.46m 1.50vw
2-O-methyl fucose	2.58w 2.86s 2.92m 3.80vw	4.05s 4.70m 5.05w
2,3,4-tri-O-methyl glucuronic acid	2.26	
2,3-di-O-methyl glucuronic acid	5.43m 6.00m 6.81s 8.50w	8.05s 8.84w 10.70m

Table 8 (cont.) Gas liquid chromatography of standard sugar glycosides and hydrolysis fractions from methylated S53

	columns (i)		columns (ii)	
Fraction I	13.5		17.5	
Fraction II	0.9s 1.19m	1.16sh 1.24w	1.05s 1.47m	1.44sh 1.50vw
Fraction III	2.55m	3.62s	3.28m	4.81s
Fraction IV	3.06m	3.50s	4.08m	4.69s
Fraction V	2.58vw 2.92m	2.86s	4.00s	4.68m
Fraction VI	1.1vw	8.4s	----	
Fraction VII	5.43m	6.6.78s	8.05s	10.66m

(s = strong m = medium w = weak sh = shoulder)

The neopentyl glycol adipate column (i) -- see 'general methods' -- was used in a Pye 104 gas chromatograph, while the polyethylene glycol adipate column (ii) was used in a Pye Argon gas chromatograph.

A methanolysate of methylated S53 polysaccharide was similarly examined on columns (i) and (ii); the resulting chromatograms are shown in figures 1 and 2. All the peaks on these chromatograms can be ascribed to the components characterised in this section, but several are in fact combined peaks formed from two sugars.

Figure 1

sugar	peaks
2,3-di-O-methyl fucose	1,2 and 3
2,4,6-tri-O-methyl glucose	4 and 8
2,4,6-tri-O-methyl galactose	6 and 7
2-O-methyl fucose	5 and 6
2,3-di-O-methyl glucuronic acid	9 and 10
X	11

Figure 2

sugar	peaks
2,3-di-O-methyl fucose	1,2 and 3
2,4,6-tri-O-methyl glucose	4 and 7
2,4,6-tri-O-methyl galactose	5 and 6
2-O-methyl fucose	5 and 6
2,3-di-O-methyl glucuronic acid	8 and 9
X	10

7. Summary of the methylation results and discussion of the methylation methods attempted.

The yields of component sugars obtained from hydrolysis of methylated S53 methylated polysaccharide are shown in Table 9.

Table 9 Yields of component sugars

Fraction	component sugar	yield (mg)	apparent percentage of original polysaccharide
I	'X'	25	1.7
II	2,3-di-O-methyl fucose	120 approx	8.0
III	2,4,6-tri-O-methyl glucose	275	18.3
IV	2,4,6-tri-O-methyl galactose	420	28.3
V	2-O-methyl fucose	140	9.3
VI	2,6-di-O-methyl glucose	23	1.5
VII	2,3-di-O-methyl glucuronic acid	116	7.8

The presence of substantial quantities (9.3%) of 2-O-methyl fucose should imply a comparable quantity of end-group sugar, but since this was not present in the hydrolysate, it would thus be likely that 'X' was in some way involved in this paradox. The striking feature of these results is the apparent absence of a recognisable end-group and the presence, albeit in small quantities, of an unidentified component 'X'.

Complete methylation of 'X' with silver oxide and methyl iodide in dimethyl formamide, followed by gas-chromatographic comparison of this methylated material with original 'X' glycoside, showed no difference in the retention time of the product. It was thus apparent that 'X' was already a fully methylated sugar and is the 'missing' end-group in our analysis; only the discrepancy in yields remained to be explained.

The preliminary investigation (part 5) had shown that 'X' was appreciable labile towards acid, and it is assumed that the somewhat longer period of time used in the hydrolysis (no detectable quantities of methylated aldobiuronic acid being obtained) also resulted in considerable degradation of the component 'X'. Examination of the gas-liquid chromatogram in figure 1 would imply that there is a much greater quantity of 'X' in methanolysates of the polysaccharide than is present in acid hydrolysates. This comparative stability to methanolysis will be mentioned in more detail in a later section, but it should be mentioned at this stage that equal quantities of 2,4,6-tri-O-methyl galactose methyl glycoside and 'X' methyl glycoside appear to give comparable responses on the PyeArgon chromatograph, such responses being measured by cutting out the peaks on the chart paper and weighing them. Figure 1 would thus indicate a large quantity of 'X' (about 10% of the methylated sugars) in the methanolysate; such an estimated content would also correspond approximately to the 9.3% of fucose branch-point and to the 10% of the native polysaccharide which could not be accounted for in the sugar analysis in Section 1.

The total sugar percentages for glucose, galactose and fucose listed in Table 9 show remarkable concordancy with the analysis of the native polysaccharide. The methylated glucuronic acid percentage is low and this is almost certainly due to degradation during hydrolysis. If we assume that the true 'X' content is about 10% and that the true methylated glucuronic acid content is about 18% prior to degradation, the total analysis is

almost exactly 100%. Making these same assumptions the proportions of constituent sugars in the methylated polysaccharide are

X	1
2-O-methyl fucose	1
2,3-di-O-methyl fucose	1
2,3,6-tri-O-methyl glucose	2
2,4,6-tri-O-methyl galactose	3
2,3-di-O-methyl glucuronic acid	2

While trace quantities of 2,6-di-O-methyl glucose may have some structural significance, it is more likely that they arise from under-methylation of the polysaccharide.

The methylation procedures using sodium hydroxide and dimethyl sulphate show marked differences according to whether the original polysaccharide solution was buffered or not. It cannot be ascertained whether degradation was by action of the alkali or by some other indeterminate factor such as the use of old dimethyl sulphate containing atmospherically-induced sulphuric acid. The lability of S53 polysaccharide in phenol extractions has already been shown (Section 1), but whether the polysaccharide has a marked instability towards alkali has not been determined. Any such lability could explain the poor yields obtained using the potassium hydroxide in dimethyl sulphoxide method.

The barium hydroxide/methyl iodide method appears to have the greatest application in the methylation of S53 polysaccharide, and it has in fact been used on all the related polysaccharides

described in the next section. Losses during methylation and work-up appear to be minimal. Completing the methylation with silver oxide and methyl iodide has the severe drawback that there is, progressive loss of material during successive methylations.

A further methylation method which has been applied to S53 polysaccharide is the sodium hydride/methyl iodide technique employed by Conrad(48), but in order to discuss certain side-reactions which are considered possible, particular structural features of S53 polysaccharide will first be elaborated in succeeding sections.

Section 3 Structural studies on other related bacterial polysaccharides

1. General description of polysaccharides examined.
2. (i) *E.coli* K12, S61 strain: extracellular polysaccharide
   (ii) *E.coli* K12, S53 strain: capsular polysaccharide
   (iii) *E.coli* K12, S53 strain: cell-wall lipopolysaccharide
3. *Aerobacter (Cloaca) cloacae* NCTC 5920: extracellular polysaccharide
4. *Salmonella typhimurium* extracellular polysaccharide
5. *Klebsiella aerogenes* 1.2, extracellular polysaccharide
6. *Klebsiella aerogenes* A3, extracellular slime
7. Summary of results.

1. General description of polysaccharides examined

From the E.coli bacterium, type K12, various strains have been developed including those which feature extracellular capsules or slimes. The E.coli K12 S53 bacterium, isolated by Dr. I.W. Sutherland of the Department of Bacteriology, Edinburgh University, can form either a slime or a capsule according to which substrain is used. The former polysaccharide has already been discussed in Sections 1 and 2, and the capsular polysaccharide will now be described. In another strain of E.coli K12 (S61), the extracellular slime is highly viscous, appreciably more so than that of S53. All three polysaccharides contain the same acid-stable component sugars in approximately the same proportions, as shown by hydrolysis and visual comparison of paper chromatograms, and all are sensitive to the same phage enzymes obtained from phage grown in an E.coli K12 host.

Apparently related to these polysaccharides is the extracellular slime from Aerobacter (Cloaca) cloacae NCTC 5920. As well as having an analogous chemical composition in terms of detectable component sugars, the polysaccharide exhibits a further relationship in its sensitivity to the phage enzymes which are found to act on the E.coli polysaccharides. In all cases, the enzymes have marked viscosity-reducing effects although the mode of action is not at this stage understood. The genus Cloaca is generally distributed in water and grasses, and only occasionally features as an enterobacterium, and the apparent chemical relationship between the polysaccharides is thus of interest.

Salmonella typhimurium 1086 also displays chemical and enzyme-response properties that relate the polysaccharide slime closely to those already mentioned.

The isolation of an unidentified methylated sugar 'X', believed to be derived from the end-group of S53 slime polysaccharide, led to an investigation of the possibility of wider general distribution of 'X', especially in the related E.coli, Cloaca and Salmonella polysaccharides. This Section will describe the methylation of these related polysaccharides and also the methylation of the slime from the non-pathogenic Klebsiella aerogenes 1.2 and the slime from the pathogenic Klebsiella aerogenes A3 (examination of which was effected prior to the publication of the work by Conrad et al (48). For further comparison, a cursory examination of the E.coli K12 S53 lipopolysaccharide. Apart from the K12 polysaccharides, the samples examined were chosen largely because of the availability of the strains in the Department of Bacteriology, University of Edinburgh. As has been mentioned above, chemical relationships were also possible in some instances.

2. (i) E.coli K12, strain S61: extracellular slime polysaccharide

A sample of the polysaccharide (250 mg.) was dissolved in water (500 ml.) and passed through a column of IR 120 cation exchange resin (H^+form). The eluate was reduced in volume in vacuo and freeze-dried. To a suspension of the polysaccharide in dry ether (50 ml.) was added an ethereal solution of

diazomethane, and the mixture was left to stand for 24 hours at room temperature. After removal of the solvent and excess diazomethane *in vacuo*, the residual esterified polysaccharide was dissolved in water and freeze-dried.

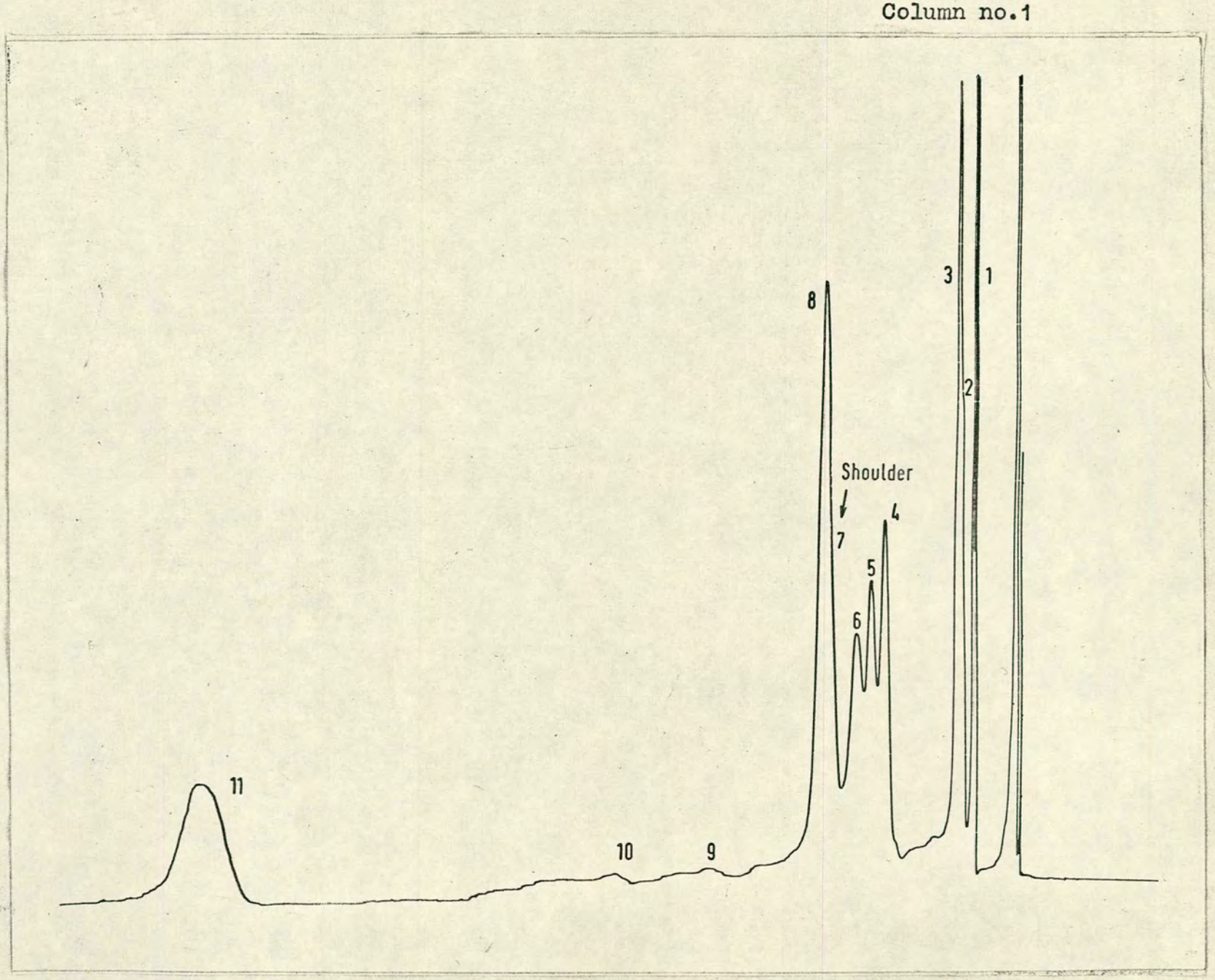
The esterified polysaccharide was dissolved with warming in dimethyl sulphoxide (30 ml.), diluted with dimethyl formamide (20 ml.) and methylated using the barium hydroxide/methyl iodide technique described on page 58. The mixture was dialysed for 72 hours against running water, the volume reduced *in vacuo*, and the solution freeze-dried.

The partially methylated material was dissolved in dimethyl formamide (25 ml.) and was further methylated in the manner described on page 59. After removal of the solvent *in vacuo* at 90°C, the residue was extracted with chloroform, and the methylated polysaccharide obtained by precipitation with light petroleum (60-80° fraction). After centrifugation, and drying at 60°C for 12 hours in a vacuum oven, the yield was 200 mg. The silver oxide treatment was repeated and the methylated S61 polysaccharide again isolated as described above. (Yield 160 mg., - OMe 38.5%)

Gas-liquid chromatography of S61 polysaccharide

Methylated S61 polysaccharide (about 5 mg) was methanolysed, neutralised, filtered and evaporated *in vacuo*. The sample was then dissolved in a small quantity of chloroform and examined by gas-liquid chromatography using columns 1 and 2 which had been used to examine fractions of S53 polysaccharide in

FIGURE 3. GAS CHROMATOGRAM OF A METHANOLYSATE OF S61 METHYLATED POLYSACCHARIDE



Section 2. The chart obtained using column 1 is shown in figure 3. Comparison with the corresponding chart for a methanolysate of S53 polysaccharide (Section 2, page 64) indicates no observable difference in the positions or sizes of the peaks obtained. The suspected end-group X is readily observable in both polysaccharides, in apparently equivalent amounts.

(ii) E.coli K12, strain S53, capsular polysaccharide

Whereas the slimes hitherto discussed are, by definition, extensive and diffuse extracellular accumulations, capsules are more compressed and sharply delimited. The capsular polysaccharide of S53 bacterium has a higher viscosity in aqueous solution than that of S53 slime or S61 slime. This might be related to the more discrete nature of the capsule in vivo.

S53 capsular polysaccharide (100 mg.) was methylated in the manner described in Section 3, 2(i), above. A small variation in technique, however, was necessary after the barium hydroxide/methyl iodide methylation stage when it was found that the freeze-dried material was not readily soluble in warm dimethyl formamide. It was necessary to pass an aqueous solution of the material through a column containing IR 120(H^+) resin and to re-esterify using diazomethane before silver oxide/methyl iodide treatment which was effected as for S61 slime polysaccharide. Yield = 81 mg; -OMe = 38.1%.

Gas-liquid chromatography of S53 polysaccharide (capsule)

A sample of methylated S53 capsular polysaccharide was methanolysed and examined on the gas-liquid chromatography columns

in the manner described above. The resulting charts were again indistinguishable from those obtained from methanolysates of methylated S53 slime polysaccharide with respect to peak positions and peak areas. The unidentified component 'X' was again present.

(iii) E.coli K12, S53 strain: cell-wall lipopolysaccharide

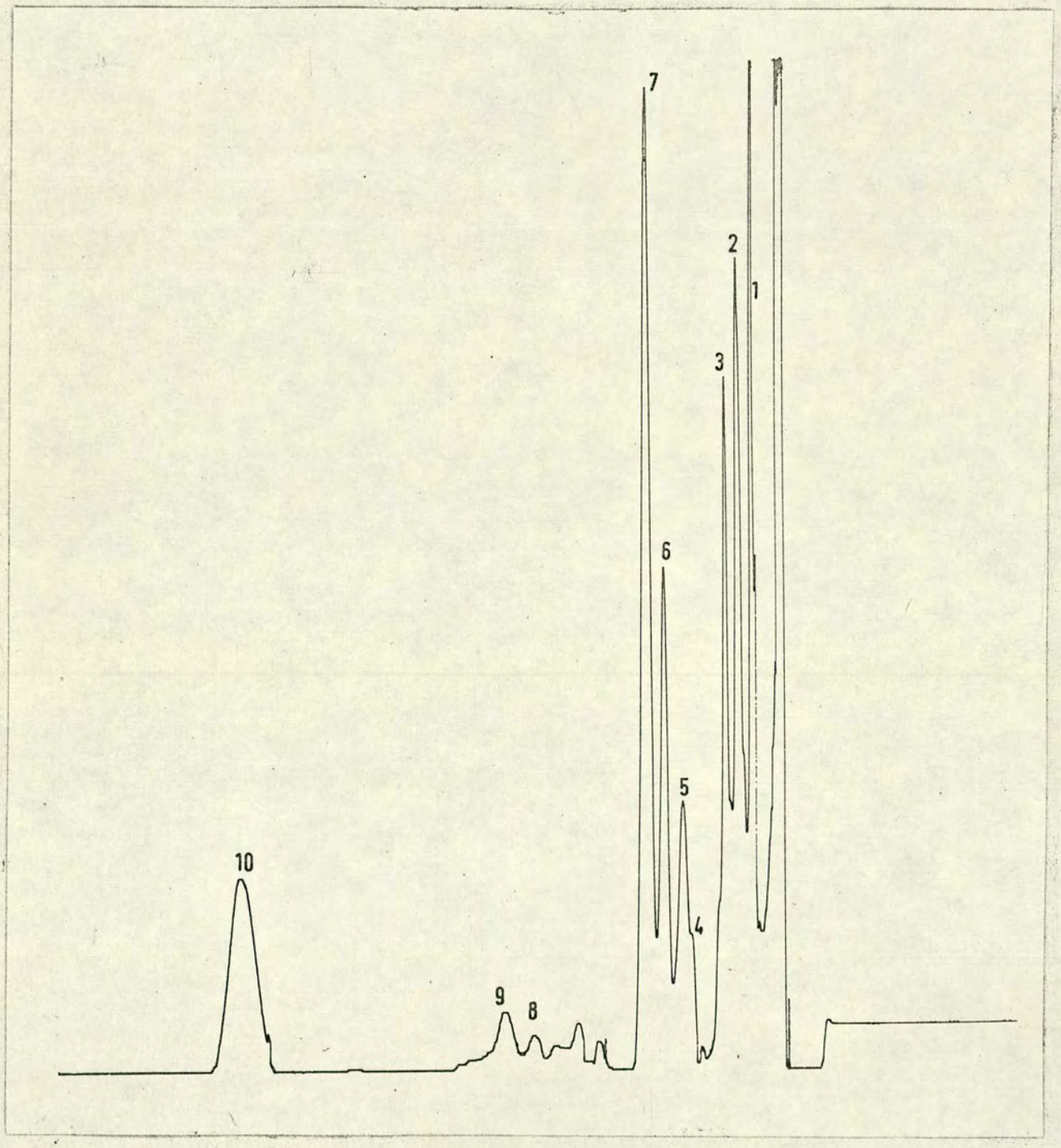
Only a cursory examination of the cell-wall lipopolysaccharide has been made. The sample was of indeterminate purity, and the nature of the component sugars has not been sufficiently examined to merit detailed investigation, but the relevant structural units have been itemised by Horecker (80). The polysaccharide was found to be soluble in dimethyl sulphoxide and was methylated directly in the manner employed for S61 slime (Section 2, part 2(i)). After methanolysis and examination on the two gas chromatography columns already described, the resulting charts showed no resemblance to these for the extracellular polysaccharides previously examined. While no attempt was made to assign peaks to particular sugars, the majority did not correspond to sugars isolated in hydrolysates of S53 polysaccharide, and there was an absence of the unidentified component 'X'.

3. Cloaca (Aerobacter) cloacae NCTC 5920, extracellular polysaccharide

After methylation and methanolysis as for the S61 slime, the C.cloacae 5920 polysaccharide gave a peak pattern identical to that obtained for the E.coli K12 capsule and slimes, including the peak corresponding to the methylated component 'X'.

FIGURE 4. GAS CHROMATOGRAM OF A METHANOLYSATE OF SALMONELLA TYPHIMURIUM POLYSACCHARIDE.

Column no. 2



4. Salmonella typhimurium 1086 extracellular polysaccharide

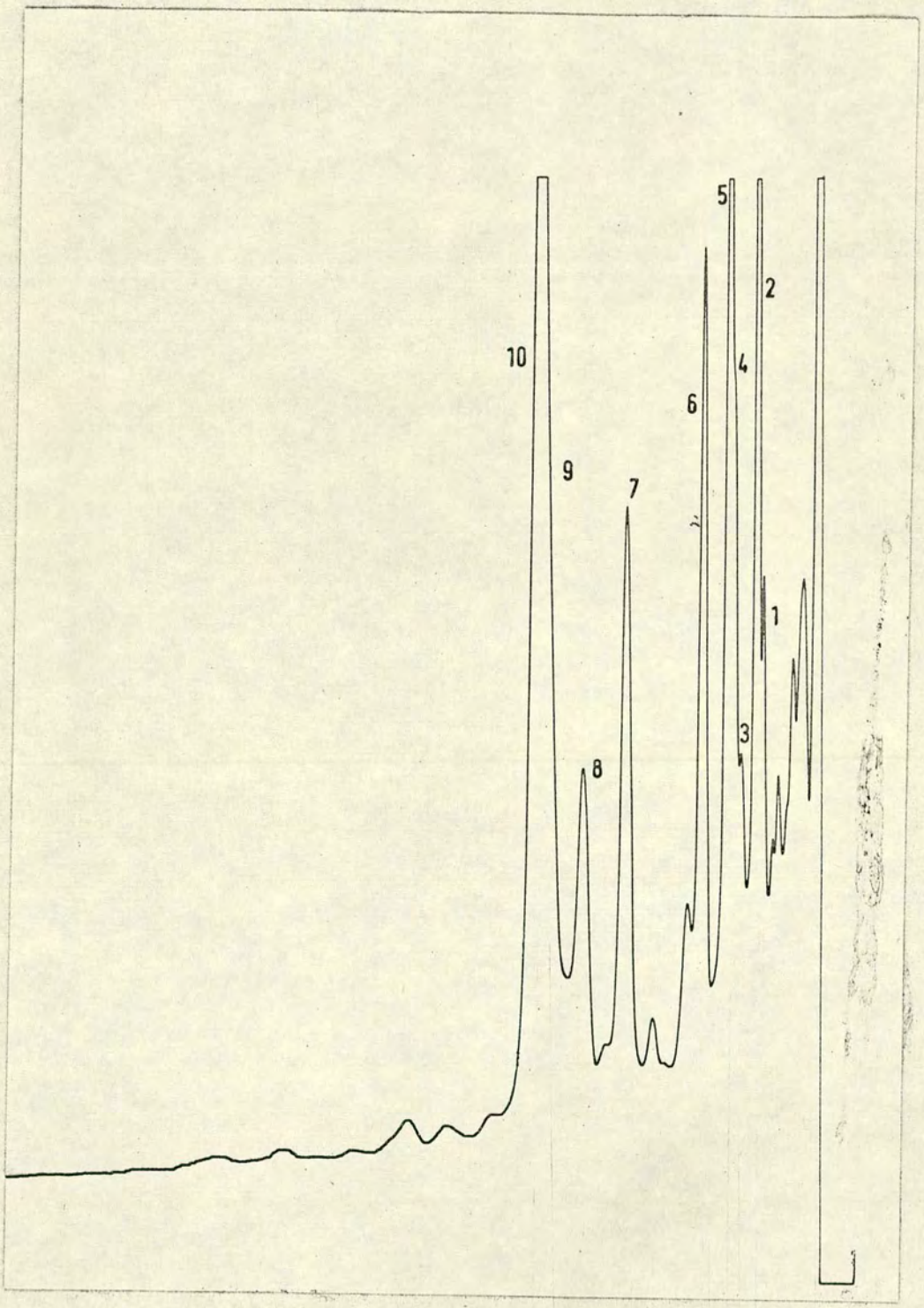
After methylation and methanolysis, the extracellular polysaccharide of this Salmonella strain was examined by gas-liquid chromatography. The chart obtained using column 2 is shown in figure 4. A number of similarities can be noticed on comparison with Figure 3, but particularly evident is a peak of relative retention time 1.81. Such a peak would correspond to 2,3,4,6-tetra-O-methyl galactose, and this identification was confirmed using column 1. Semi-quantitative comparison of the peak areas of X (relative to one of the 2,4,6-tri-O-methyl galactose peaks) in this polysaccharide and S53 polysaccharide -- as measured by weighing the cut-out paper peaks -- showed an apparent decrease in X content of about 15%. The experimental error of this weighing method is estimated at about 10% from control experiments. Peak 4 in figure 4 (of relative retention time 3.10) corresponds to the major peak of 2,3,6-tri-O-methyl galactose, the minor peak having a retention time of 3.50, and this peak might represent undermethylated galactose end-group; traces of slower, undermethylated products can be seen on the chart.

5. Klebsiella aerogenes 1.2, extracellular polysaccharide

A preliminary analysis of the exopolysaccharide from Klebsiella aerogenes 1.2 indicated the presence of glucose, galactose, fucose and glucuronic acid units. The proportions were similar to those in S53 polysaccharide except that rather

FIGURE 5. GAS CHROMATOGRAM OF A METHANOLYSATE OF KLEBSIELLA 1.2 POLYSACCHARIDE

Column no. 2.



less galactose was present (personal communication by Dr. I. W. Sutherland, Dept. of Bacteriology, University of Edinburgh). The polysaccharide was methylated using the barium hydroxide/methyl iodide method already described, and a sample was methanolysed, neutralised and examined in chloroform solution by gas-liquid chromatography. The resulting chromatogram for column 2 is shown in figure 5, and the retention times of peaks are listed below.

peak	retention time
1	0.87
2	1.05
3	1.31
4	1.44
5	1.48
6	1.90
7	3.13
8	3.81
9	4.42
10	4.52

Reference to table 8 shows that these peaks can be assigned as follows:

sugar	peak(s)
2,3,4-tri-O-methyl fucose	1
2,3-di-O-methyl fucose	2, 4 and 5
2,3,4,6-tetra-O-methyl galactose	6
2,3,6-tri-O-methyl galactose	7, 8 and 9 (part)

The sugar corresponding to the minor peak 3, and the sugar(s) corresponding to peak 10 and part of peak 9 could not be identified with the standards available, although the absence of derivatives of glucose amongst the identified methyl ethers would suggest that 9 and 10 might be derived from 3,4,6-tri-O-methyl glucose.

Column 1 gave similar information but also revealed a peak of retention time of 10.70 which might correspond to 2,4-di-O-methyl galactose. The absence of 'X' on both chromatograms was noted.

A sample of the methylated polysaccharide (30 mg.) was hydrolysed in 45% formic acid (100°, 4 hours) and the acidic components isolated by preparative electrophoresis. Elution with alcohol and evaporation to dryness was followed by paper chromatography in solvent B. and development of spots with p-anisidine hydrochloride. Only one spot, corresponding to 2,3-di-O-methyl glucuronic acid standard, was observed.

No further investigation of this polysaccharide was carried out.

6. Klebsiella A3 slime polysaccharide

Prior to the published results of Conrad et al (48, 49), A3 slime polysaccharide had been methylated using the method adopted for the polysaccharide of E.coli K12, S61. Examination of a methanolysate of the methylated material by gas-liquid chromatography had resulted in the identification of 2,3-di-O-methyl glucuronic acid, 2,3,4,6-tetra-O-methyl glucose and

a di-O-methyl fucose, although this could not be identified with the standards available. Paper chromatography in solvent F of a hydrolysate of the methylated polysaccharide, followed by development with p-anisidine hydrochloride, had showed a spot with similar characteristics to that of 2,3-di-O-methyl fucose, but the necessity or desire for further investigation of the polysaccharide was obviated by the publication by Conrad.

7. Summary of results

In the extracellular slimes and capsule of the E.coli K12 bacterium, there is a striking apparent constancy in their chemical compositions, including the presence in the methylated polysaccharides of the unidentified component 'X'. The polysaccharide of Cloaca cloacae 5920 also appears to share this common chemical structure, and the Salmonella typhimurium polysaccharide, although not identical, appears to possess a close similarity. The last-named might, from a biosynthetic viewpoint, be termed "incomplete". The E.coli lipopolysaccharide, however, was completely lacking, after methylation, in the component 'X', indicating that this sugar does not occur generally throughout the immediate environment of the cell-wall. Neither has 'X' been found as a component of the methylated ethers of any of the other polysaccharides examined.

This close similarity in sugar composition, enzyme susceptibility (see Section 5) and methylation pattern, especially

with regard to the existence of the unidentified sugar X, is remarkable, and lends weight to the view expressed by several authors that related species of bacterium may elaborate a common polysaccharide. The immense differences in viscosity of these different polysaccharide solutions would still, however, require explanation, and particularly the fact that the natural biological state may vary from a loose slime to a firm capsule.

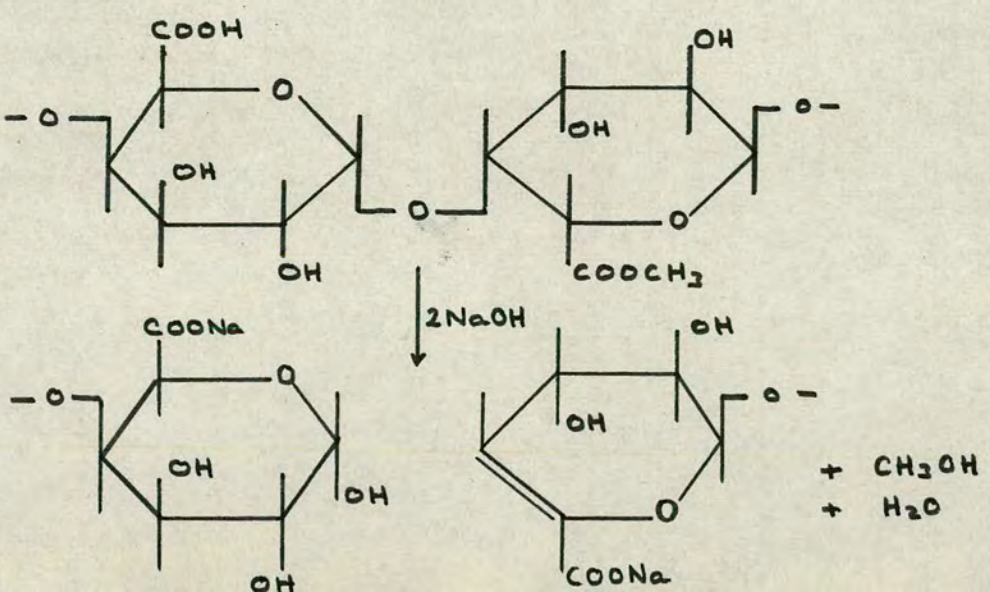
Section 4 The degradation of S53 polysaccharide by anhydrous base; the detection of a hitherto unknown monosaccharide, and the relationship of this sugar to the methylated moiety 'X'.

1. Introduction
2. Esterification of S53 polysaccharide
3. Fragmentation of the ester using sodium methoxide in methanol
4. Examination of the aqueous acetone-soluble fraction by paper chromatography and by methylation.
5. Examination of the aqueous acetone-insoluble fraction by paper chromatography and methylation.
6. Methylation of the water insoluble polysaccharide residue.
7. Base-catalysed fragmentation of methylated polysaccharide.
8. Methylation of S53 polysaccharide with sodium hydride and dimethyl sulphate.
9. Summary and discussion of results.

1. Introduction

The alkaline degradation of polysaccharides using sodium hydroxide has long had industrial significance in, for instance, the treatment of wood pulp for the paper and rayon industries. Richards and Sephton (81) reported the isolation of D-glucoiso-saccharinic acid from the oxygen-free treatment of cellulose with boiled 0.5N sodium hydroxide, and it is generally accepted that this and many other reported degradations by alkali proceed from the reducing end-groups by a step-wise elimination of monosaccharide residues. Glycosidic linkages within the chains are extremely alkali-resistant, although the oxidation of adjacent hydroxyl groups sharply diminishes this resistance.

The instability of esters of pectic and alginic acids during alkaline saponification, however, was explained by Neukom and Deuel (82) as being due to a β-elimination reaction, basing their mechanism on earlier work by Kenner (83). According to this mechanism, the glycosidic linkage in the β-position to the ester carbonyl group of pectin is cleaved following the removal of the activated hydrogen at C_5 and the formation of a C_4-C_5 double bond.



The non-esterified carbonyl group at C6 is not sufficient to activate the hydrogen at C_5 in alkaline solution, and sodium pectate is thus not affected in this manner. Haug, Larsen and Smidsrod showed that the β-elimination of alginates proceeded to a minor extent at pH's on the acid side of neutral.

The alkaline extraction of certain polysaccharide components from natural material raises the question as to whether these components are true constituents or whether they are part of some larger molecule degraded by β-elimination in the course of the extraction. The paper by Rees and Richardson (84) summarises previously published examples by other workers that such degradation does occur -- e.g. the 'pectic araban' described by Barrett and Northcote (85), and describes experiments on a mustard-seed araban which confirm it to be a genuine constituent of the seeds.

In addition to chemical degradation of polysaccharides by β-elimination, there are several reports of enzyme systems capable of producing this same breakdown. Thus alginic acid can be degraded using a Pseudomonad enzyme or hepatopancreas eliminases (86 and 87) while the determination of the fine structure of hyaluronic acid has been assisted by selective eliminases (88).

2. Esterification of S53 polysaccharide

The esterification of S53 polysaccharide in the H^+ form has been described in Section 2, part 3a, and the same technique was employed in esterification of material prior to β-elimination, using propylene oxide instead of ethylene oxide. As was indicated

in Section 2, the pH of the mixture always reached a maximum value of about 4.9, and it was found that this could be improved if the solution was reduced in volume by two thirds on a rotary-film evaporator and fresh propylene oxide added. After a week at room temperature, the pH was found to rise to about 5.5. Dialysis for several days followed by freeze-drying gave ester in 65-70% yield.

The use of diazomethane for esterification of polysaccharides has the disadvantage that the reaction is heterogeneous and is likely to give rise to methyl ethers as well as methyl esters, especially if the esterification conditions are prolonged in an attempt to obtain complete esterification. Rapid esterification is an advantage of this method, but the experiments described below have involved the use of propylene oxide esters only.

3. β-elimination of S53 polysaccharide ester

(i) sodium methoxide in methanol method

Using a method based on work carried out by Mr. J.W.B. Samuel, (Chemistry Dept. University of Edinburgh) S53 polysaccharide ester (1g) was suspended in a mixture of methanol (40 ml) and 2,2-dimethoxypropane (5 ml) -- the latter was water-scavenger -- and shaken overnight in a stoppered flask. Another flask containing methanol and dimethoxypropane in the same quantities was left standing overnight; to this was added dry sodium (40 mg) which was allowed to dissolve completely. After 15 minutes, the sodium methoxide solution was added to the polysaccharide suspension, the flask was re-stoppered, and the mixture was shaken for 24 hours.

The solution acquired a noticeable yellow colour during this period, and a sample of the mixture was found to give strong absorption at 235 mμ on an ultra-violet spectrometer, such absorption being characteristic of α,β-unsaturated esters.

Excess sodium methoxide was neutralised with N HCl and the solvents removed on a rotary film evaporator. To the residue was added water (50 ml) and the flask was shaken for 4 hours, a substantial portion of the residue remaining undissolved. The mixture was filtered through glass paper, the insoluble material was repeatedly washed with further water, and the combined filtrates were reduced in volume on a rotary-film evaporator to about 5 ml. The water-soluble material was fractionated by addition of acetone (50 ml), the precipitated material being separated by centrifugation and washed with 90% acetone solution. The supernatant solution was evaporated to dryness in vacuo, and the aqueous acetone-insoluble and water-insoluble residues were dissolved in water and freeze-dried. Yields were 120mg, 350mg and 600mg respectively.

4. Examination of the 'aqueous acetone-soluble' fraction

Preliminary paper chromatography indicated, as expected, that a proportion of this fraction consisted of salts. These were removed by passing an aqueous solution through IR 120 (H^+) and IR 45 ion-exchange resins, and the eluate was reduced in volume on a rotary-film evaporator. Samples of this solution were examined before and after treatment with borohydride using the phenol-sulphuric acid method, and the lack of response of the reduced

material was taken to indicate that any components of this fraction were monosaccharides only. On evaporating to dryness by co-distillation with a 1:1 mixture of ethanol and benzene, 35 mg of light-brown residue were obtained. Samples of this material were examined by paper chromatography, the results being indicated in Table 11.

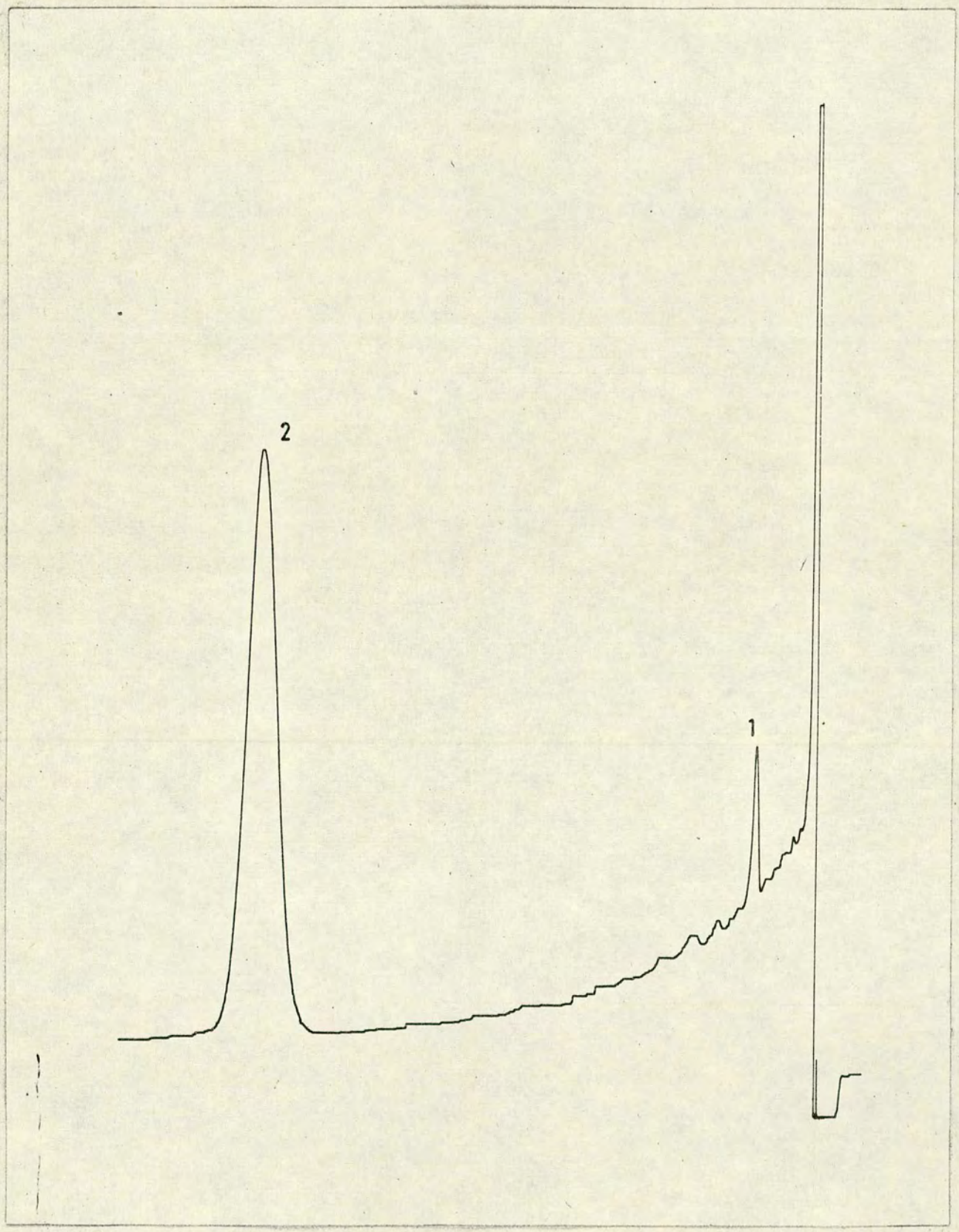
Table 11 Paper chromatography of the acetone-soluble fraction

sugar	R_G in solvents	
	A	B
fucose	1.7	1.6
main component of aqueous acetone-soluble fraction	2.05	1.9

In both solvent systems only one spot was observable which gave a brown coloration with p-anisidine hydrochloride spray. Reference to Section 1 will show that this monosaccharide is not evident in acid hydrolysates of native S53 polysaccharide, and a later Section will deal with some of its properties.

A relationship between this monosaccharide and the methylated moiety 'X' was by now suspected, and the acetone-soluble fraction was accordingly methylated using the silver oxide/methyl iodide in dimethyl formamide technique, the early part of the methylation being carried out at 0°C. The mixture was diluted with a large excess of chloroform and left to stand for several hours, after

FIGURE 6. GAS CHROMATOGRAM OF THE METHYLATED, AQUEOUS-ACETONE SOLUBLE MATERIAL AFTER ALKALINE FRAGMENTATION



which the chloroform supernatant was washed several times with water, dried over anhydrous sodium sulphate, and evaporated to dryness on a rotary-film evaporator. The light brown residue was methanolysed in 3% methanolic hydrogen chloride for 6 hours at 80°C and the neutralised solution was examined on a gas-liquid chromatograph using columns 1 and 2. The resulting chromatogram from column 1 is shown in figure 6. The major component is a peak corresponding to the slow-moving, unidentified, methylated component 'X', while trace quantities of tetra-O-methyl galactose are also observable. An increase in the sensitivity of the apparatus also reveals even smaller quantities of tri-O-methyl fucose. Hydrolysis of the glycoside(s) using 30% formic acid at 100°C for 2 hours, followed by paper chromatography in solvent and development with p-anisidine spray, gave a brown spot of the same R_f value as was obtained with the component X from methylated polysaccharide, although considerable streaking was evident, possibly associated with partial degradation.

5. Examination of the fraction insoluble in aqueous acetone

Paper chromatography of an aqueous solution of this fraction using solvent E and spraying with p-anisidine failed to detect any oligosaccharides apart from a faint response on the starting line, and it was concluded that it was of comparatively large molecular weight. Phenol-sulphuric acid determinations on original and borohydride-reduced samples appeared to show no detectable difference

in colour response, indicating that the average degree of polymerisation was probably greater than 10.

The freeze-dried material was soluble in N-methyl-2-pyrrolidone, and a sample (50 mg.) was accordingly methylated in this solvent using the barium hydroxide/methyl iodide method. The mixture was diluted with chloroform and left overnight, the chloroform supernatant being then filtered and washed several times with water. The solution was evaporated to dryness on a rotary-film evaporator and left overnight over P_2O_5 in a vacuum oven. The partially methylated material was then completely methylated using silver oxide and methyl iodide in dimethyl formamide, working up the methylation in the usual manner. Precipitation of a chloroform solution of the methylated material using light petroleum was not successful, and the material was therefore isolated as a syrup by evaporation. Yield 38 mg, -OMe = 39.9%.

A sample was methanolysed, neutralised and examined by gas-liquid chromatography, the resulting chromatograms being identical to those obtained from methanolysates of intact methylated polysaccharide, except for an additional peak in both the chromatograms from colums 1 and 2. This additional peak occurred at a retention time of 0.70 on neopentylglycol adipate and at 0.80 on polyethylene glycol adipate, and reference to Table 8 would indicate that this component is 2,3,4-tri-O-methyl fucose. Hydrolysis of a sample of the methylated oligosaccharide and paper chromatography in solvent G showed a component with the properties of 2,3,4-tri-O-methyl fucose.

The extent of fragmentation was variable, presumably depending on the extent of esterification of the polysaccharide. In other experiments carried out subsequently, smaller fragments appear to be present in the oligosaccharide mixture.

6. Examination of the water-insoluble polysaccharide residue

The water-insoluble residue, which formed the bulk of the product after fragmentation, was totally insoluble in all solvents tried. The use of urea and sodium lauryl sulphate also failed to assist the solution of this intractable material in water, and it was therefore macerated in a blender with N-methyl-2-pyrrolidone and methylated in this finely suspended form using the barium hydroxide/methyl iodide method over 4 days. After dialysis and freeze-drying, a surprisingly high yield of 200 mg from 250 mg of starting material was obtained. This material was completely methylated using the silver oxide/methyl iodide in dimethyl formamide method, and a sample of the methylated material methanolysed and examined on a gas-liquid chromatograph. The resulting chromatogram was almost identical to those obtained from methanolysates of methylated S53 polysaccharide apart from a marked dimunition in the quantity of 'X'. Hydrolysis of a sample of the water-insoluble fraction, and paper chromatographic comparison with hydrolysates of native polysaccharide, indicated an appreciable decrease in the amounts of glucuronic acid in the former.

7. Base catalysed fragmentation of methylated polysaccharide

While fragmentation of the ester of the native polysaccharide is useful as a method of obtaining a monosaccharide component which does not survive acid hydrolysis, the reaction is heterogeneous and is seems likely that the extent of reaction is limited by accessibility of the -COOH groups. The use of methylated polysaccharide, which is soluble in methanol, provides a homogeneous reaction, and it was hoped that further information on the fragmentation products might be obtained, especially if the reaction would go to completion. Methylated polysaccharide (25mg) was dissolved in methanol (10 ml) and 1 ml of dimethoxypropane added, the solution being left for 3 hours. Sodium methoxide solution (10 ml), prepared as in part 3 above, was added and the mixture left overnight. The mixture, which had acquired a faint yellow colour, was neutralised with dilute hydrochloric acid and evaporated to dryness on a rotary-film evaporator. The residue was dissolved in 1 ml of chloroform and to this was added dropwise with shaking light petroleum (20 ml). The precipitated material was centrifuged off, washed with more 85% light petroleum, evaporated to dryness and methanolysed. The supernatant solution was similarly evaporated to dryness and methanolysed. Both samples were examined by gas-liquid chromatography. The precipitated material gave a chromatogram which was similar to that from methylated polysaccharide apart from a dimunition of the size of the 'X' peak to about one half. The supernatant solution gave a similar chromatogram except that here there was appreciably more 'X' than would have been expected. It seems that some

unprecipitated material identical to that in the precipitated fraction was present in the supernatant solution.

8. Sodium hydride methylation of S53 polysaccharide

The sodium hydride methylation technique used by Conrad et al (see Introduction to this thesis) might possibly have β-elimination side-reactions once the carboxyl groups of polysaccharides became esterified. In the case of S53 polysaccharide, it is apparent that such side reactions would result in a reduction in the amounts of 'X' in methanolysates of hydride-methylated polysaccharide.

Methylated S53 polysaccharide (25mg), esterified by passing through a column of Amberlyst 15 and treatment with diazomethane (-OMe 39%) was dissolved in dry dimethyl sulphoxide. The solution was treated with sodium hydride powder (25mg), whereupon the solution turned a dark brown (DMSO itself did not do this), and after one hour, methyl iodide (5 ml) was added and the mixture stirred for 16 hours during which time the depth of colour diminished to give a golden solution. Excess hydride was destroyed with methanol and the solution diluted with chloroform, washed with water (5 x 100 ml), dried over sodium sulphate, and evaporated to dryness. The residue was dissolved in chloroform (1 ml) and light petroleum, (50 ml) added, the solution being then left overnight. The resulting precipitate was centrifuged off, washed, and dried. The supernatant solution was evaporated to dryness to yield 1 mg of residue, which might have included impurities from solvents. No difference could be discerned between the precipitate and ordinary methylated

polysaccharide by methanolysis and gas chromatography, but the supernatant fraction was found to contain some 'X'. High sensitivities were required to detect this peak, so that the quantity must have been small.

In a repeat experiment, the residue after methylation was dissolved in spectroscopic alcohol and examined for absorption at 235mu. Such absorption was detected, but it has been found that dimethyl sulphoxide absorbs in this region, and the possible presence of traces of dimethyl sulphoxide, in spite of washing chloroform solutions with water, cannot be discounted.

As a final experiment with sodium hydride, S53 polysaccharide ester (100 mg) was dissolved in dimethyl sulphoxide (20 ml) and sodium hydride (200mg) added in small portions. After 1 hour, methyl iodide (5 ml) was added over a period of 45 minutes, and the mixture was left stirring for 16 hours. Addition of reagents was repeated and the mixture left for a further 16 hour period. Excess hydride was destroyed with methanol and the reaction mixture was worked up as above. Precipitation of a chloroform solution of the residue with light petroleum yielded 75 mg of methylated material, and methanolysis and examination of this by gas-liquid chromatography showed no discernable difference in peak pattern as compared with S53 polysaccharide methylated by the barium hydroxide method.

9. Summary and discussion of results

The most striking result from these experiments is the detection of the monosaccharide component which, in the methylated form, appears as the unidentified component 'X' in methylated S53 polysaccharide. A later section will show that of many methods attempted, alkaline fragmentation has proved to be the only way that this new monosaccharide can be isolated. The remarkable mobility in paper chromatography of this sugar both in its normal and methylated forms, together with its high retention times on gas chromatography, imply that this new sugar has certain structural features not normally found in naturally-occurring monosaccharides. Until more information regarding its structure is reported, this monosaccharide will be, pro tem, termed 'chrisnose'.

Because fragmentation probably occurs by the β-elimination mechanism, and since the nature of the aldobiuronic acid is known, the following partial structure of the polysaccharide may be proposed:

chrisnose $^{1}\longrightarrow{}^{4}$glucuronic acid $^{1}\longrightarrow{}^{3}$galactose ^{1}.....

Because oligosaccharide fragments are also formed in this cleavage reaction, it seems that the glucuronic acid also exists in other positions. The variable degree of polymerisation of the fraction suggests that it contains a spectrum of molecules which vary in size, smaller molecules resulting from efficient fragmentation, larger molecules being the result of only intermittent fragmentation.

The unsophisticated fractionation technique employed after

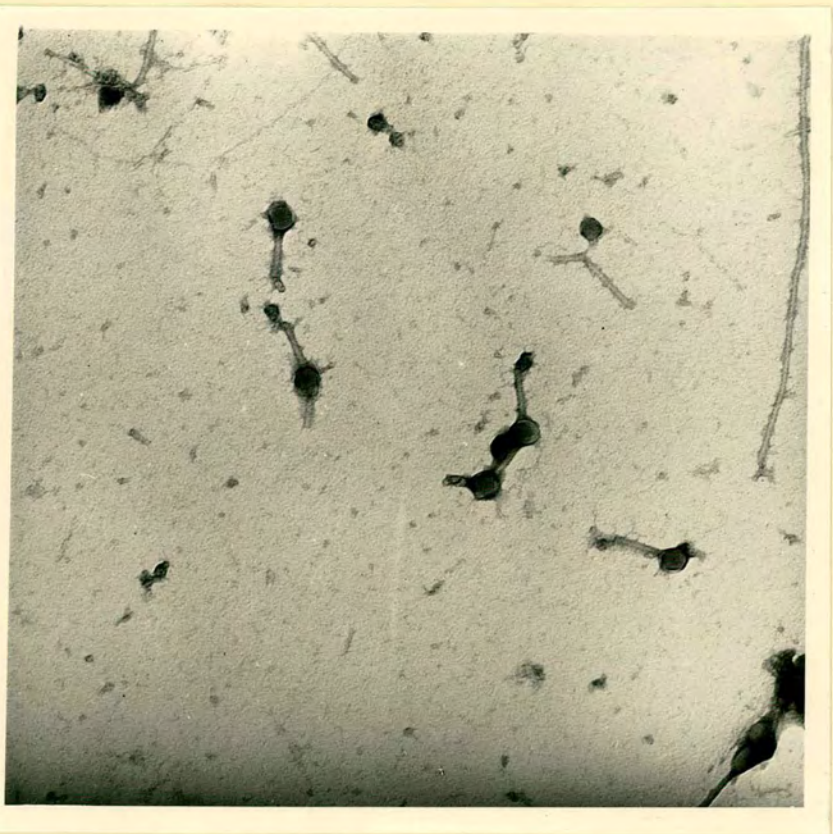
alkaline fragmentation of the methylated polysaccharide renders detailed discussion impracticable. The extent of this elimination was apparently very much lower than had been expected in view of the homogeneous nature of the reaction, but the concentration of X in one fraction as against a lower concentration in the other, shows that some elimination had occurred.

The sodium hydride methylation of native polysaccharide ester would appear to have been at least as successful as other methods and probably more convenient. Elimination, although perhaps occurring to some extent in the hydride treatment of methylated polysaccharide ester, appears to have been minimal or nil. This would be in agreement with the results obtained by Conrad et al in their methylation of the Aerobacter aerogenes A3 slime polysaccharide.

Section 5 The action of bacteriophage enzyme on E.coli K12 extracellular polysaccharides

1. Introduction
2. Isolation of bacteriophage
3. Viscosity and sedimentation characteristics of K12 polysaccharides before and after enzyme attack.
4. The nature of the reducing end-groups produced by enzyme attack.
5. The methylation of the enzymically-degraded polysaccharides, and examination by gas chromatography.
6. Discussion

FIGURE 7. ELECTRON MICROGRAPH OF F5 PHAGE



1. Introduction

The infectious particle of a typical phage, (see the electronmicrograph of F5 phage shown in figure 7), consists of a polyhedral head to which is attached a short, thinner tail. The head has an outer coat of protein which encloses a core of nucleic acid; the tail consists only of protein and has a hollow core. During the process of adsorption, the tail of the particle becomes attached to the bacterial wall, and by virtue of a specific enzyme in the tail, creates a small perforation at the site of attachment. Injection of the deoxy-ribonucleic acid and subsequent take-over of the genetic machinery of the cell to produce more phage can now be accomplished.

It is obvious that a viscous slime or capsule affords considerable physical hindrance to the attachment of the phage tail to the bacterial cell-wall. While there is evidence that the phage tail may contain some enzymic material capable of depolymerising this slime, it is generally accepted that the bulk of phage depolymerase is synthesised within the bacterial cell by phage which have successfully injected their DNA, the lysis of such cells resulting in the release of depolymerase and the breakdown of further slime material prior to full-scale phage attack.

Wilkinson and Sutherland (91) isolated a range of bacteriophages grown on E.coli K 12 or on Cloaca cloacae 5920, and showed that the isolated phage preparations were specific for the strain upon which they were grown, the phages cultivated on E.coli K 12 being avirulent with respect to C.cloacae 5920 (apart from one

phage which may have wider virulence). The action of the isolated depolymerase enzymes, however, was much more general, and these were capable of reducing the viscosity not only of polysaccharide from the host strain but also of polysaccharides of the same apparent chemotype from other strains and species. The authors point out that the production of a common mucoid substance by several species of enterobacteriaceae has been previously suggested, and such generality of enzyme action was thus not unexpected.

2. Isolation of bacteriophage

Using the procedure of Wilkinson and Sutherland, Edinburgh sewage (400 ml.), sterile broth (200 ml.) and, chloroform (300 ml), were shaken vigorously for several minutes, stood 3-4 hours at 0°C, and the clear upper layer used without further treatment for bacteriophage isolation.

Nutrient broth cultures (10 ml.) of the potential host (cultivated overnight at 37°C) were diluted 1:10 with fresh sterile broth and incubated for two hours at 37°C. To these were added 1 ml samples of the sewage preparation and the mixture incubated overnight at 37°C. The cultures were centrifuged briefly at low speed to remove most of the bacterial cells, and the supernatant solution was added to sterile vials (1 oz., screw cap) and heated at 60°C for 30 minutes to kill any remaining host bacteria. Ten-fold dilutions in saline were made, and 0.1 ml samples from each dilution were spread over nutrient agar plates which had been flooded with a culture of E.coli K12 S53 bacteria and allowed to

dry. After incubation at 37° for 24 hours, the plates were examined for discrete plaques, material representing each plaque type being removed with a sterile needle and re-inoculated into 2-hour cultures as above. The procedure was repeated until particular dilutions showed only one plaque type, this being assumed pure.

Using the above method, a culture of phage type F1 was isolated, and large amounts of this were prepared by the soft agar-layer technique. 100 ml nutrient broth samples were inoculated with S53 bacterium and incubated at 37° overnight. The sample was poured into 500 mls. of sterile semi-solid agar -- the latter at a temperature of about 45°C -- and about 5 ml of phage preparation added, the resultant mixture being poured onto nutrient agar trays and incubated overnight at 37°C. The semi-solid agar was poured from the trays and centrifuged at low speed, the supernatant being then dialysed overnight and reduced in volume in a cold-room using polyethylene glycol. After concentration, the dialysis tubing was placed in phosphate buffer (0.02M, pH = 7.2) for two hours, the mixture centrifuged at 20,000 rpm in a refrigerated centrifuge for 15 minutes, and the solution (the crude enzyme preparation) stored in a refrigerator prior to further purification.

Adding saturated ammonium sulphate solution gave a series of fractions from 10-90% saturation, the bulk of polymerase being obtained between 40-60% saturation. This fraction was redissolved in the phosphate buffer and chromatographed on a DEAE-cellulose

FIGURE 8. GRAPHS SHOWING THE VISCOSITY/TIME RELATIONSHIPS FOR ENZYME-POLYSACCHARIDE SYSTEMS.

Curve A 0.1 ml enzyme solution

Curve B 0.5 ml enzyme solution

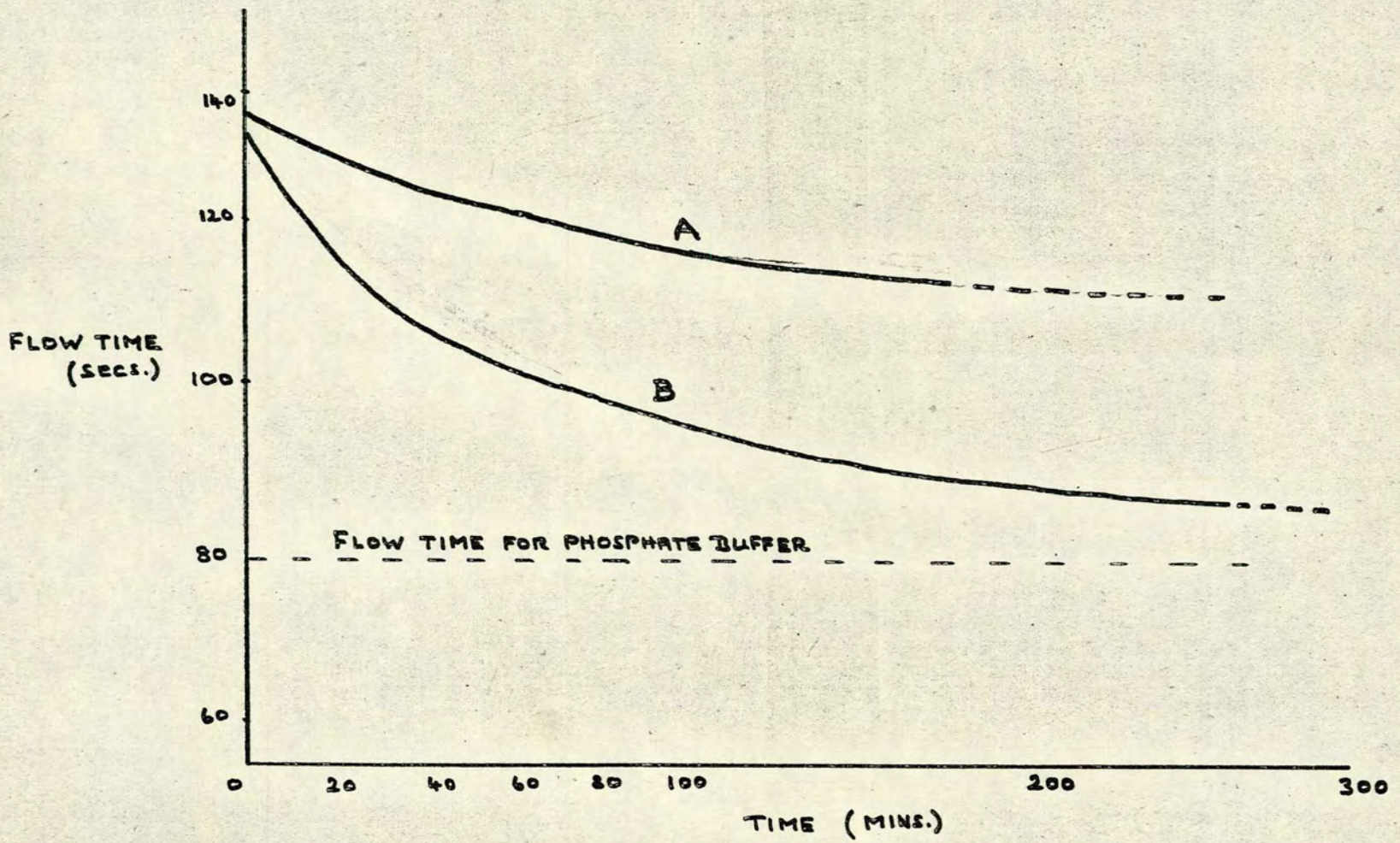


Table 13 Flow after addition of 0.5 ml of enzyme preparation

Time (mins)	Flow time (secs)
0	130
3	127
6	124
9	121
12	120
30	108
70	98
140	90.5
270	86.5
20 hours	81

Graphs are drawn in figure 8 relating viscosity against time for the above systems, and it can be seen from these that the ultimate viscosity of the polysaccharide solutions is very near to that of phosphate solution alone. Ths viscosity change over the first 100 minutes is 26% for the sample containing 0.1 ml of enzyme preparation and 71% for the sample containing 0.5 ml.

To confirm the completeness of enzyme attack, a 0.5% solution of the polysaccharide solution in phosphate buffer was incubated at 25° for 4 days with 1 ml of enzyme preparation, the minimum viscosity time dropping to 101 seconds compared with 80 seconds for phosphate buffer blank. Addition of a further 0.5 ml of enzyme preparation had no further effect on the viscosity

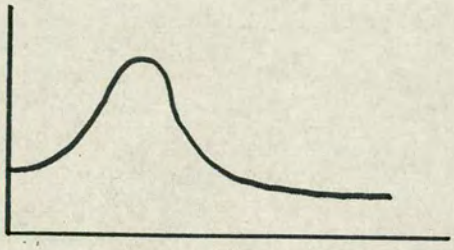
of the solution.

Incubation of solutions of S53 capsular polysaccharide and of slimes of S61 and 5920 strains with enzyme, gave similar results. Incubation of S53 slime polysaccharide at 37° resulted, as expected, in a more rapid reduction in viscosity of the solution, a minimum value being obtained after 28 hours as compared with 60 hours at 25°.

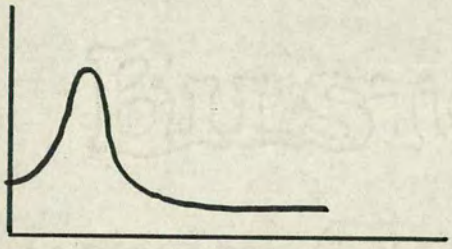
For subsequent experiments described in this section, S53 polysaccharide was treated with enzyme in the following manner. Polysaccharide (0.8g) was dissolved in phosphate buffer (0.02M, pH = 7.2)(100 ml) and penicillin and streptomycin sulphate (100 mg each) added. To the solution was added enzyme preparation (3 ml), and the mixture was incubated at 37° for 2 days. During this period, the turbid solution became clear, and the viscosity diminished. Small scale experiments, and the results of Wilkinson and Sutherland, had already indicated that the product of enzyme attack was non-dialysable, and the solution was accordingly dialysed overnight, reduced in volume on a rotary-film evaporator and freeze-dried, the yield being 0.8g.

Samples of the original polysaccharide and of the enzyme degraded material were examined in the ultracentrifuge (kindly effected by Mr. I.W. Steele) at 59,780 rpm on 1% solutions the resulting sedimentation diagrams after 76 minutes being shown in figure 9.

Figure 9 Sedimentation diagrams



S53 polysaccharide



enzyme-degraded polysaccharide.

The sedimentation rates would seem to be of the same order, suggesting comparatively minor change in molecular weight, but complications arise in assessment of the sedimentation rate of the original polysaccharide molecules owing to the viscosity of the solution.

4. The nature of the reducing end-groups produced by enzyme attack

Enzyme-degraded polysaccharide (200 mg) was dissolved (very readily) in water (100 ml), and potassium borohydride (50 mg) added. After 72 hours, the excess borohydride was destroyed using formic acid, and the solution made 40% with respect to the acid and hydrolysed overnight at 100°. The hydrolysate was worked up in the usual manner to remove formyl esters, and examined by paper chromatography in solvents 1 and 2. No apparent decrease in the

amounts of any of the sugars was apparent when compared with hydrolysates of native S53 polysaccharide.

A portion of the hydrolysate was passed in turn through ion-exchange resin columns of IR 120 (H^+) and IR 400(OH^-), the latter column being washed with several bed-volumes of conductivity water to elute sugar alcohols. Concentration of this eluate, and paper chromatography of the product in solvent system 4 followed by development of spots with periodate/benzidine reagent, gave a single spot in the sample corresponding to that of standard glucitol, the reducing group revealed by enzyme attack thus being that of glucose.

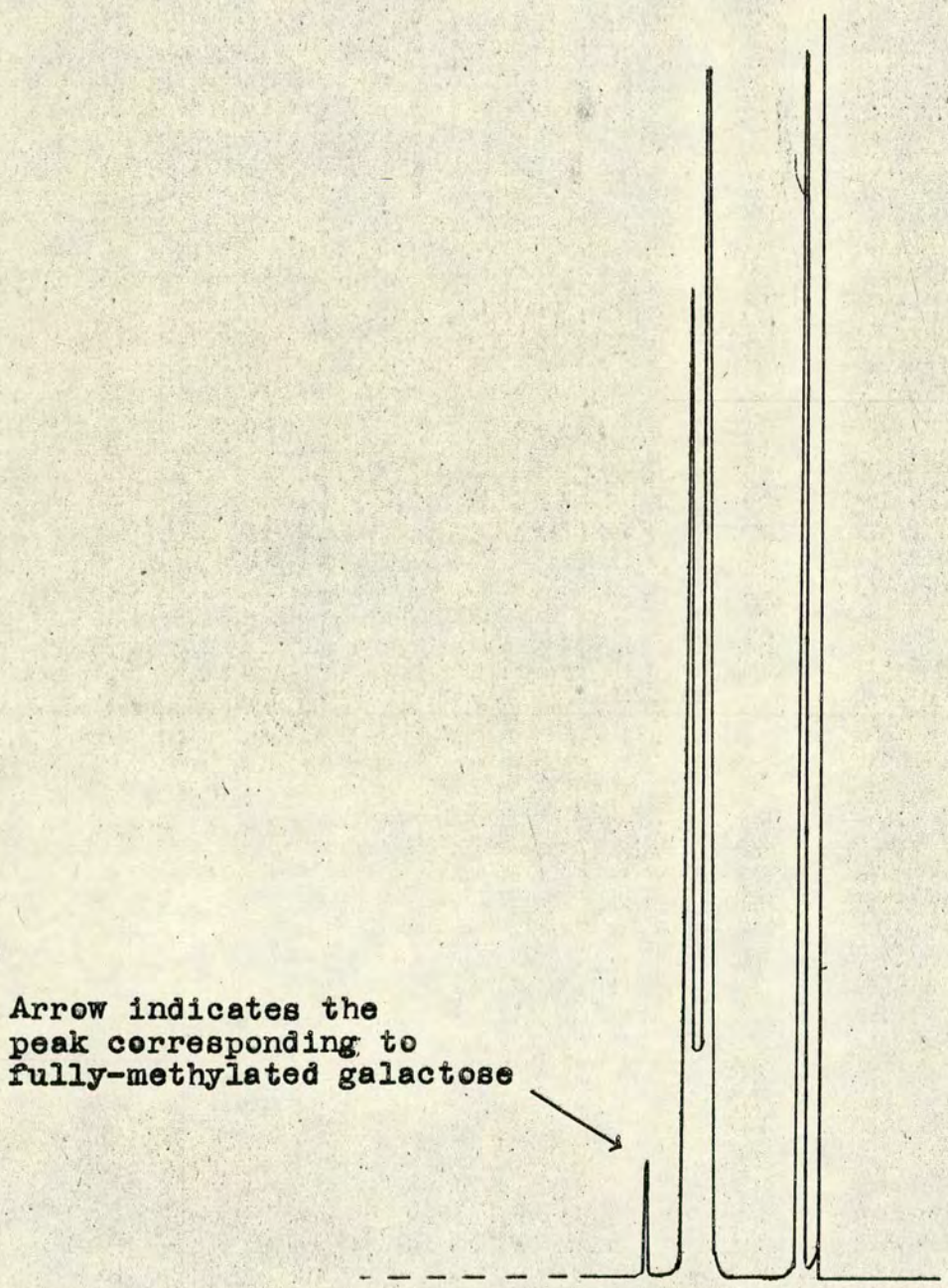
A portion of the formic acid hydrolysate dissolved in water was similarly passed through columns of IR 120 and IR 400 (formate) resins, and acidic sugars eluted from the latter using formic acid. Removal of formic acid and electrophoresis in pyridine/acetic acid buffer (pH = 7.5) showed only the presence of glucuronic acid and traces of aldobiuronic acid when the spots were developed using periodate/benzidine reagent. Chromatography gave the same results. It was thus concluded that L-gulonic acid is not present in the hydrolysate of borohydride-reduced material, but later evidence that galactose may constitute the non-reducing end group requires possible revision of this result.

5. <u>The methylation of the enzymically degraded S53 polysaccharide and examination of the product by gas-liquid chromatography</u>.

The degraded polysaccharide was found to be somewhat sparingly soluble in N-methyl-2-pyrrolidone without prior

FIGURE 10. GAS-LIQUID CHROMATOGRAM OF A METHANOLYSATE OF METHYLATED, ENZYME-DEGRADED S53 POLYSACCHARIDE.

(fast-moving components only shown)



esterification, but 200 mg could be dissolved in 100 ml of the solvent. Barium hydroxide/methyl iodide methylation was performed in the usual manner, and the mixture dialysed and freeze-dried. This partially methylated material (175 mg) was further methylated with silver oxide and methyl iodide and the mixture diluted with a large excess of chloroform and left to stand overnight. The chloroform supernatant was washed several times with water, dried over sodium sulphate, and reduced in volume on a rotary-film evaporator. The addition of light petroleum did not induce precipitation of the methylated polysaccharide, and this was therefore isolated as a syrup by evaporation. After drying overnight over phosphorus pentoxide in a vacuum dessicator at 60°, a yield of 140 mg was obtained.

After methanolysis and neutralisation, a sample was examined on a gas-liquid chromatograph using columns 1 and 2. The resulting chromatograms were almost indistinguishable from those obtained from methanolysates of methylated S53 polysaccharide with the exception of small peak in both chromatograms corresponding to 2,3,4,6-tetra-O-methyl galactose. The size of this peak can be seen in figure 10 which shows the peaks corresponding to 2,3-di-O-methyl fucose in this sample, together with the minor peak, which corresponds to the fully methylated galactose.

6. Discussion of results

The action of phage enzyme on S53 polysaccharide gives rise to no dialysable products and yet results in a marked reduction

in the viscosity of the solution. This indication that enzymic cleavage is only of limited extent would appear to be confirmed by the results of the sedimentation experiments.

The use of borohydride showed the formation of glucose reducing-groups, and the detection of small quantities of tetra-O-methyl galactose in the methylated degraded polysaccharide, presumably arising from new end-groups, would indicate that the action of the enzyme is to cleave certain glucosyl-galactose linkages in the core of the polysaccharide.

Section 6 Investigations into the structures of 'X' and of 'chrisnose'

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D. Summary of results

A. Introduction

Hydrolysis of the methyl ethers of several related polysaccharides had yielded a methylated sugar 'X' with high paper-chromatographic mobility, yet with very long retention time on the columns used for gas chromatography. Treatment of this methylated sugar with methyl iodide and silver oxide did not result in any change in properties, and the lack of any other recognisable end-group was further evidence that this unidentified component was a terminal unit in the methylated polysaccharides examined.

Alkaline fragmentation of the native polysaccharide ester, presumably through a β-elimination mechanism, had resulted in the production of a monosaccharide component not detectable in acid hydrolysates of S53 polysaccharide. Methylation of this paper-chromatographically fast-moving material resulted in the formation of 'X', a relationship between 'chrisnose' and 'X' being thus clearly established.

This Section will describe some of the investigations which have been carried out regarding the structure of these sugars.

B. Studies on X

1. a) Purification of the methylated sugar

It has already been indicated that extended hydrolysis of methylated polysaccharides invariably resulted in diminished quantities of the sugar X, presumably owing to acid degradation.

Isolation of X as the free sugar by this method has therefore been used very little. Purification was effected by cellulose-column chromatography in solvent H followed by preparative paper chromatography in the same solvent. The sugar was eluted from the paper using water.

b) Purification of the methyl glycosides of X

Methanolysis of methylated polysaccharides (3% methanolic hydrogen chloride, 100°, 4 hours) yielded a mixture of glycosides which contained substantial proportions of methyl glycosides of X. It was ultimately found that the most convenient way to obtain pure X glycosides was to completely methylate the methanolysate using silver oxide and methyl iodide in dimethyl formamide; conversion to completely methylated sugars greatly assisted the chromatographic resolutions described below.

i) alumina column chromatography

A mixture of fully-methylated glycosides (1g) was dissolved in ether (20 ml) and applied to a column of B.D.H. activated alumina (bed-volume 50 ml) which had been equilibrated with the same solvent. Slow elution with ether was accompanied by gas-chromatographic examination of the eluate. After a considerable period of time, a fraction containing X glycosides with approximately 50% of other material was obtained. Rechromatography did not markedly improve the purity of this fraction, but this treatment served as a useful preliminary purification.

ii) silica-gel column chromatography

The partially purified material (0.4g) was applied to a column of silica gel, again equilibrated with ether. Elution with the same solvent produced much better and sharper resolution than had been obtained with alumina, the fraction containing X glycosides being contaminated with about 10% of other material the bulk of which appeared to be 2,3-di-O-methyl fucose probably arising from undermethylation of the original methanolysis mixture. The silica-gel column chromatography was repeated using an ether-benzene 6:1 system, and the X-glycosides were obtained in a pure state as shown by gas chromatography. A later fraction contained the 2,3-di-O-methyl fucose together with further small quantities of X glycosides.

iii) attempted preparative gas-liquid chromatography

Prior to column chromatography, it had been considered likely that particularly high retention times of X glycosides in column chromatography would lend themselves to purification by this technique. However, not only did preparative gas-liquid chromatography (column 4, 225°) fail to give more than minor separation, but upon examination of the 'eluate' using columns 1 and 2, a major peak with a retention time approximately one half that of X itself was evident. The poor resolution using this preparative gas-chromatography may have simply been a result of using columns of poor condition; it is possible that the marked degradation was a result of using the necessary high temperatures

coupled with the effect of the hot aluminium surface in which the column material was packed.

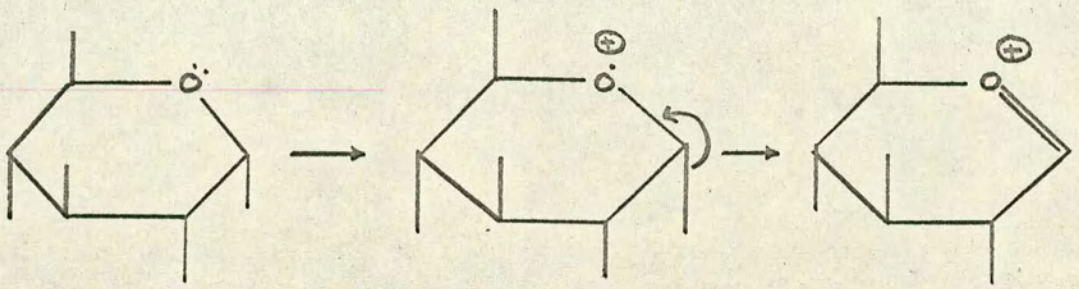
iv) Thin-layer chromatography on silica-gel

Resolution of the product into what were assumed to be the two anomers was achieved very successfully using preparative, thin-layer, silica-gel chromatography. Using large plates, 10 mg samples of the glycoside material were spotted in ether solution and the plate developed with the ether-benzene 6:1 solvent system. The two bands were detected using iodine vapour and were scraped off the plate into suitable containers, the material from each band being eluted with ether and examined as described below. Using a carefully equilibrated silica-gel column, as described in (ii) above, separation of anomers could similarly be accomplished. A substantial portion of the eluate, however, consisted of a mixture of the anomers, resolution being less efficient.

2. Gas-liquid chromatography of the methyl glycosides

A feature of X in its methyl glycoside form has been its high retention time on gas chromatography columns 1 and 2. These columns have appreciable polar characteristics, however, and it was found that non-polar columns such as SE 52 (column 3) not only gave much lower retention times at a temperature of 150°C but could also partially resolve the pure fraction of X glycoside into two peaks (retention times 4.10 and 4.25 on

FIGURE 11 C_1-elimination pathway



m/e = 219

TABLE 14 The major fragments in mass spectrometry of X-glycosides

m/e		
247	97	69
155	95	59
145	88	57
127	85	55
117	81	45
113	75	43
111	73	39
99	71	

column 3). Using the same column, the two fractions obtained on preparative thin-layer plates above were each found to give a single peak, the faster thin-layer material having a retention time of 4.10, and the slower thin-layer material having a retention time of 4.25. XE 60 (column 4) was also capable of resolving this fraction but to a lesser extent.

3. Molecular weight determinations

a) by mass spectrometry

A review of Kochetkov and Chizhov (92) has indicated the increasing importance of mass spectromery as a technique for elucidating the structures of carbohydrates. Degradation of a fully methylated methyl hexopyranoside can occur via two initial pathways, elimination at C_1 or elimination at C_5; the former degradation pathway would commence in the manner shown in figure 11.

A sample of each X-glycoside was kindly examined on a low-resolution mass spectrograph by Dr. W. Kelly, Unilever Research Laboratories, Sharnbrook, Bedford. The resulting spectrograms were sufficiently similar to confirm the assumption that they were, in fact, glycosides of the same sugar. Major peaks were observed at m/e values listed in table 14 opposite. Some of the fragments at lower m/e values are common to the literature spectra of methylated methyl hexopyranosides and furanosides. The largest fragment from X glycosides had a mass

of 247. Since methyl glycosides rarely display a detectable parent-ion peak, the largest fragment is usually formed by loss of the glycosidic methoxyl group (figure 11), and it seemed likely that the molecular weight of the original glycosides was 247 + 31 = 278. High resolution mass spectrometry showed that this largest fragment had a mass of 247.11535, such a mass ruling out all possible elemental constitutions other than $C_{11}H_{19}O_6$ for this fragment. Accurate measurement of the mass of some of the smaller fragments (155.06999 and 127. 07538) similarly revealed the presence of only carbon, hydrogen and oxygen.

Simple arithmetic shows that a fragment of molecular formula $C_{11}H_{19}O_6$ is, in mass terms, equivalent to the fragment obtained from a tetra-O-methyl hexoside (219) plus a carbon and an oxygen atom (219 + 28 = 247).

b) Vapour phase osmometry

The apparatus used for this experiment was a Mecrolab 301.A. Two identical glass beads support a drop of solvent and a drop of solution respectively, and the beads are then suspended in an atmosphere saturated with solvent. The rate of evaporation of solvent from (or the rate of condensation on) these beads is proportional to the molarity of the solute. Differing evaporation or condensation rates between the two beads and recorded as temperature-differences which are then related to the molarity of solute.

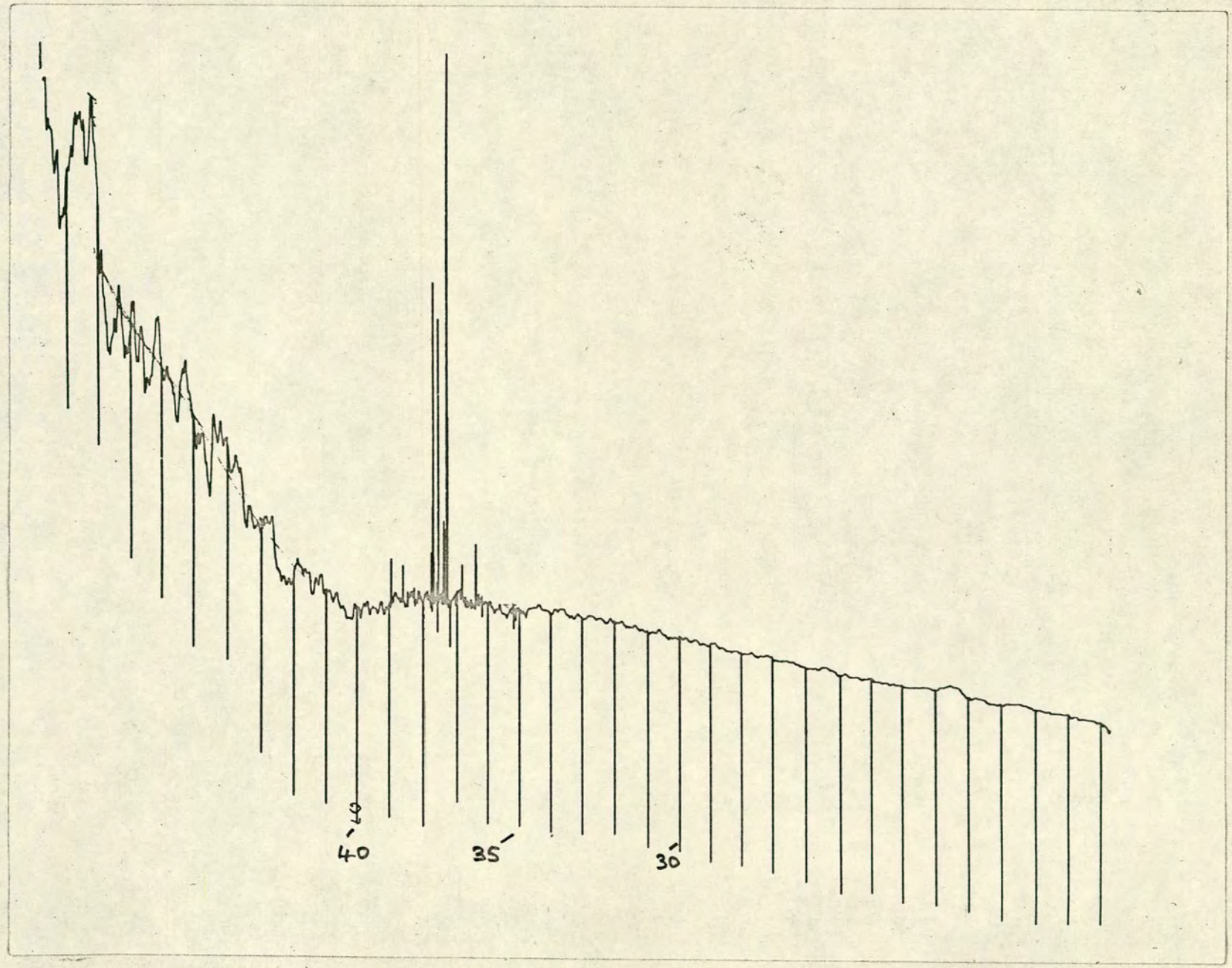
Using chloroform as solvent and benzidine as calibration compound, the molecular weight of a mixture of X glycosides was determined as 286. If the true molecular weight is 278, the osmometry figure represents a 2.9% error on a machine allegedly accurate to 2%. Other workers in this Department place a somewhat less generous value (about 5%) on the accuracy of the determination.

c) from elemental analysis

Duplicate samples of the glycoside mixture analysed as follows:-

	C	H	O (by difference)
1	51.21	7.17	41.62
2	51.47	6.94	41.59

FIGURE 12 O.R.D. spectrum of X glycosides



Wave-numbers indicated as subscripts

The empirical formula from these results being

1. $C_{12}H_{19}O_7$ or $C_{13}H_{22}O_8$
2. $C_{12}H_{19}O_7$

The above results are in agreement with the indications from mass-spectrometry that the molecular formula is $C_{12}H_{22}O_7$.

4. Infra-Red spectrum

A sample of the mixture of X glycosides was examined as a solid film on an infra-red spectrophotometer, a strong absorption peak at 1755 cm^{-1} being observed. This corresponds to a ketone carbonyl in a 5-membered ring or to an ester.

5. Optical rotatory dispersion measurements.

The magnitude of optical rotations varies with the wavelength of the light used, such variation being known as optical rotatory dispersion. When the measurements are extended into the region of an optically active absorption band, curves showing maxima and minima are observed, and these represent the Cotton effect. This occurs at the point of maximum absorption, and this fact has been used to attempt a differentiation between a ketone and an ester in X glycosides. In addition, the sign of the Cotton effect can and does assist in assigning a particular structure to a molecule by comparison with analogous compounds, but the remarkable lack of esoteric bacterial monosaccharides in the Department does not encourage speculation in this field.

FIGURE 13 P.m.r. spectrum of X glycoside

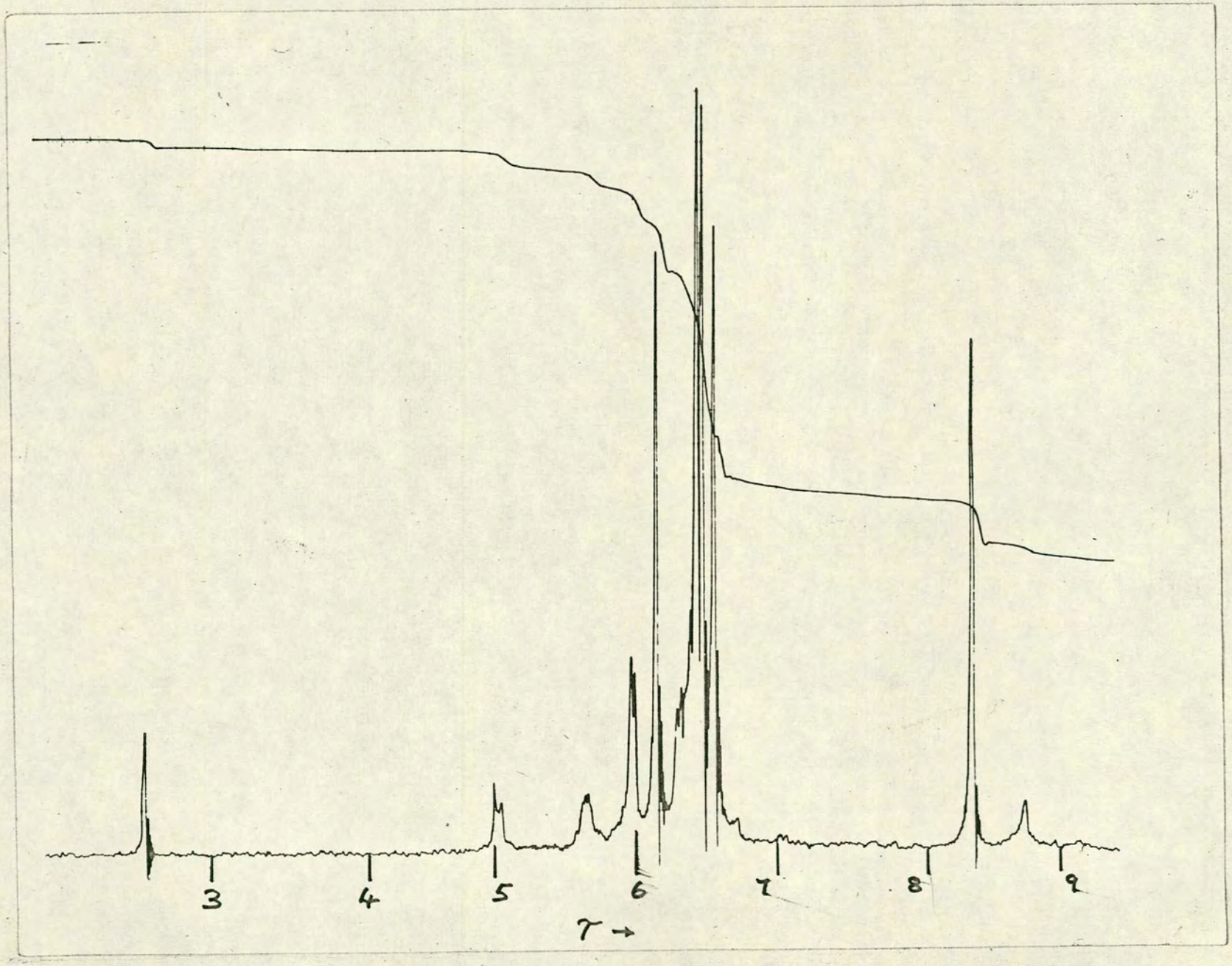


Figure 12 shows the spectrum obtained for the X glycosies. A Cotton effect (possibly positive) occure at 40-41 wave-numbers (about 247 mμ), a similar Cotton effect at 247 mμ being observed with standard methyl ester-glycoside of 2,3,4-tri-O-methyl glucuronic acid. No Cotton effect was observed around 300 mμ, the region which would be expected for a ketone. The indications are, therefore, that X contains an ester group, although it would be desirable to check this conclusion using circular dichroism because the Cotton effect in the above experiment is small compared to the background rotation, and it would be unwise to ignore the possibility that it is an artefact.

6. Proton magnetic resonance spectrocopy

The p.m.r. spectrum of the chromatographically faster-moving X-glycoside in $CDCl_3$ is shown in figure 13. There are six large singlets at τ values of 5.15, 6.45, 6.48, 6.49, 6.58 and 8.38. A doublet at about 5 τ is assumed to represent the anomeric proton, whence the integral indicates that each of the major singlets corresponds to 3 protons, i.e. with one exception, probably methoxyl groups. A singlet of particular interest, however, is the peak at 8.38 τ which would appear to be due to a C-methyl group; the p.m.r. spectrum of rhamnose in D_2O gives a similar singlet at 8.47 τ. The results can thus be summarised as 1 ester methoxyl (6.15 τ)

4 ether methoxyls (6.45, 6.48, 6.49, 6.58 τ)

(presumably including the glycosidic methoxyl)

and 1 C-methyl group (8.38 τ)

Without further knowledge of the partial structure of X-glycoside, little further interpretation of the spectrum is of practical value. There may, however, be some significance in the existence of a high-field proton at 8.74 τ.

7. Chemical experiments

a) ozonolysis

The possibility of unsaturated linkages in X glycoside was investigated. A sample (5 mg) was dissolved in chloroform and ozone passed through the solution for one hour. Subsequent gas chromatography on column 3 showed no change in retention time.

b) treatment of the methyl glycosides with formic acid

A sample of X glycoside (15 mg) was dissolved in warm formic acid (90%, 1 ml) and water (1 ml) added dropwise. In separate experiments, samples were heated at 60° overnight and at 100° for 24 hours, the hydrolysates being evaporated to dryness. The resulting electropherogram which was identical in each case is shown in figure 14, the bulk of the anisidine-positive material (red spot) being electrophoretically immobile. Paper chromatography in solvent H showed that most of the material was much slower-moving than X would have been, an

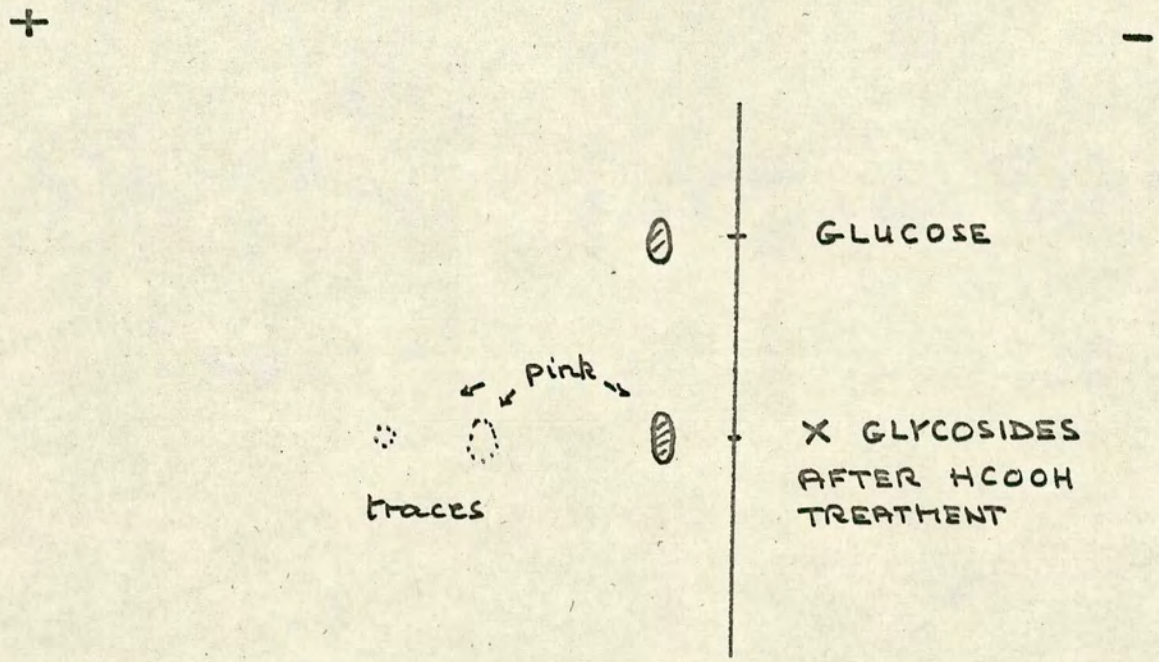


FIGURE 14 Electropherogram of product after treatment of X glycosides with formic acid.

'R_X' figure of about 0.3 being obtained. Methanolysis of the products from each hydrolysis still yielded X glycosides as shown by gas chromatography.

c) Treatment of the methyl glycosides with base

In addition to the silver oxide/methyl iodide methylation technique, monosaccharides can also be completely methylated using barium hydroxide and dimethyl sulphate (93). However, treatment of X glycosides with these reagents in dimethyl formamide solution resulted in the complete disappearance of X from gas chromatograms after 48 hours treatment at room temperature. This was eventually traced to the action of the base, and the results using various base systems are shown below.

barium hydroxide/dimethyl sulphate

A sample of X glycosides (about 5 mg.) was dissolved in dimethyl formamide (1 ml), and barium hydroxide octohydrate (50 mg) and dimethyl sulphate (0.1 ml) added at 0°C. The mixture was shaken at 0° for an hour and at room temperature for a further 48 hours, a sample of the mixture being removed after 24 hours, treated with ammonia (S.G. 0.88, 0.5 ml), and diluted with 20 ml of chloroform. The 48 hour mixture was similarly treated. The supernatant chloroform solutions were washed several times with water and dried over anhydrous sodium sulphate. After reduction in volume, samples were examined by gas-liquid chromatography using column 3. The 24 hour sample was found to contain small quantities of X together with

very much larger quantities of material which gave a peak with retention time approximately 1.4 x that of X glycosides. No other peaks were observed. The 48 hour sample displayed neither X not the other compound but gave a chromatogram consisting of a very great number of low 'humps'.

Control experiments with acid-free dimethyl sulphate and with barium hydroxide confirmed that the latter compound was the one responsible for the disappearance (and ultimate degradation?) of X glycosides.

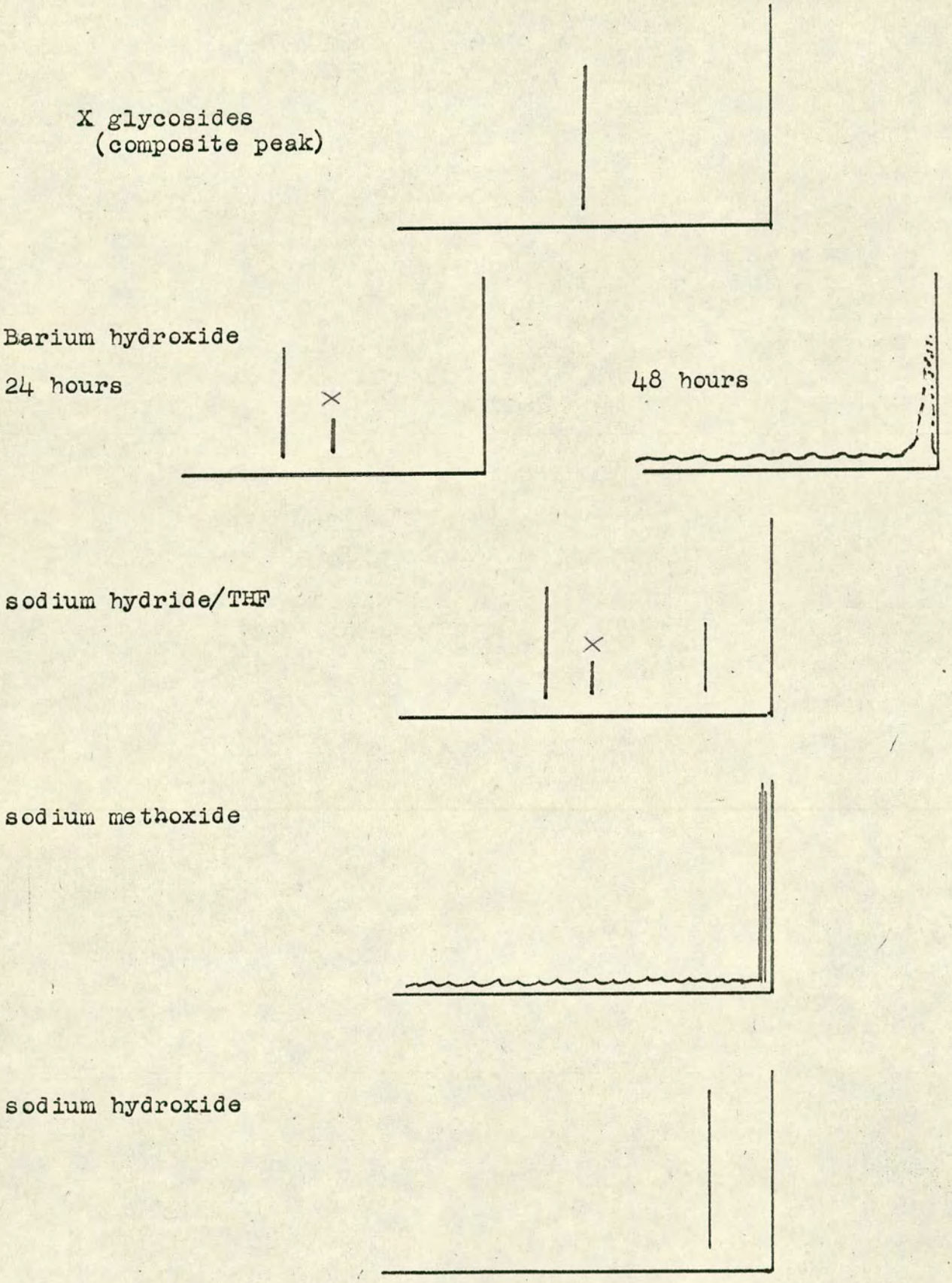
sodium hydride in tetrahydrofuran

A sample of X glycosides (about 5 mg) was dissolved in dry tetrahydrofuran (5 ml.) and sodium hydride (20 mg) added in small portions. The mixture was left for 24 hours and treated with 2 ml of methanol to remove excess hydride. The solution was diluted with water (5 ml) and extracted with chloroform (3 x 10 ml), the chloroform extracts being dried and reduced in volume prior to examination on a gas chromatograph using column 3. The major product was a peak of retention time 1.4 relative to X glycosides (small quantities of which were present), probably corresponding to that obtained after 24 hours treatment of X glycosides with barium hydroxide. In addition, a smaller peak of retention time 0.25 relative to X was also observed.

sodium methoxide in methanol

Using 2,2-dimethoxy propane, as water scavenger, a solution of sodium methoxide in methanol was prepared in the same manner

FIGURE 15 Diagrammatic representation of gas-liquid chromatograms obtained after treatment of X with various base systems.



as in alkaline fragmentation experiments in Section 4. To a sample of X glycosides (about 5 mg) was added 1 ml of this solution, and the mixture was heated at 60° for one hour. After careful neutralisation of the cooled solution with dilute hydrochloric acid, the solution was evaporated almost to dryness and the residue extracted with several washings of chloroform. The combined chloroform extracts were reduced in volume and examined by gas-liquid chromatography as above. Substantial quantities of fast-moving material and a generally 'untidy' looking chromatogram were inferred to indicate that degradation had occurred.

Sodium hydroxide

A portion of X glycosides was dissolved in methanol and treated with 0.5 N sodium hydroxide in 20% aqueous methanol at room temperature for 4 hours. After neutralisation with hydrochloric acid, the solution was reduced in volume and extracted with chloroform, and the dried chloroform, and the dried chloroform extracts were examined on the gas chromatograph as above. A single peak of retention time 0.22 relative to the X peak was evident.

The results of the action of various bases are summarised in diagram form in figure 15.

d) Colour reactions

The absorptions of specific chromophores produced in colour reactions can be used to differentiate between different

types of sugars, and such colour reactions have been employed in investigations of the sturcture of chrisnose. Part C deals more specifically with these colour reactions. With the L-cysteine/sulphuric acid reagents, X gave λ_{max} = 395 mμ, and with phenol/sulphuric acid reagents λ_{max} = 475 mμ. Both these values are typical of the chromophores derived from methylated hexoses, methylated heptoses, octoses, etc½. absorbing at quite different wavelengths.

e) Attempted demethylation

X (about 10 mg) in dichloromethane (2 ml) was cooled in acetone-cardice, and similarly cooled boron trichloride (0.5 ml) added, the mixture being kept at -80° for 30 minutes and afterwards at room temperature for 16 hours. Anhydrous conditions were maintained throughout. The solvent was removed in vacuo and resual boric acid removed by formation of volatile methyl borate in co-distillation with methanol. Paper chromatography gave no recognisable spots after development with p-anisidine, streaking associated with extensive degradation being evident instead. A sample of D-glycero-D-gulo heptose was treated in a similar manner, and degradation was found to be minimal.

Part C Studies on chrisnose

8. Attempted isolation of chrisnose by methods other than alkaline fragmentation

Acid hydrolysis of S53 polysaccharide was shown in Section 1 to yield glucose, galactose, fucose, glucuronic acid an glucurone only. In the light of the later conclusions that chrisnose was an end-group, acid hydrolysis using 0.05N sulphuric acid and partial hydrolysis using N sulphuric acid for varying short periods of time at 100° were attempted as methods of obtaining the sugar from native polysaccharide. Various oligosaccharide fractions were obtained but no trace of the acid-labile component chrisnose was at any time detected. Samples of the partial hydrolysates were neutralised and evaporated to dryness, then methylated with silver oxide and methyl iodide at 0°C for 2 hours then at room temperature for 2 days. Gas-liquid chromatography failed to reveal any peaks which would correspond to X. Partial acid hydrolysis, using formic acid, was similarly unsuccessful. Acetolysis was attempted using acetic anhydride, glacial acetic acid and sulphuric acid in the ratio of 2:1:0,5 at 0-5° for one hour then at room temperature for 2 days. Samples were removed at regular intervals and worked up in the usual manner (95). De-acetylation was accomplished in dry methanolic sodium methoxide in a cold room overnight, water was added to dissolve the precipitated material, and the mixture was neutralised through a column of IR 120 (H^+) resin. Chromatography revealed large quantities of oligosaccharide

material but a conspicuous absence of chrisnose.

9. Molecular weight from vapour-phase osmometry

Using D-glucose for calibration purposes, the molecular weight of chrisnose was determined in aqueous solution as 198 and 215 on consecutive runs. Difficulty was encountered in this experiment since chrisnose proves to be very hygroscopic to the point of deliquescence. The figure of 198 was obtained using material which had been dried in a vacuum over at 40° overnight, while the latter figure was from material which had just been removed from the freeze-drier. The figure of 215 is thus more likely to be relevant.

10. Treatment of chrisnose with formic acid

A sample of the sugar (about 5 mg.) was dissolved in 5% formic acid solution (1 ml.) and heated in a small stoppered test-tube at 100°C. Using a micropipette, samples were removed at 0, 5, 15, 30, 60 and 120 minutes, spotted onto chromatography paper and allowed to dry in a stream of cold air. When dry, one drop of water was added to each spot to destroy formyl esters, and the spots again allowed to dry before chromatography in solvent C followed by development of spots using p-anisidine. The light brown spot corresponding to chrisnose, unaffected at zero time, was completely replaced after 5 minutes by a 'streaky' spot moving with an R_F very slightly faster than that

fucose standard. No change in the appearance of the chromatogram was evident after longer periods of hydrolysis.

The experiment was repeated using 1% formic acid solution, and while chrisnose could still be detected after 40 minutes hydrolysis, visual comparison of spot intensities indicated that the half-life of the sugar was about 15 minutes using this dilution of formic acid.

11. Periodate oxidation of chrisnose

a) Total comsumption of periodate (96)

Consumption of periodate can be followed spectrophotometrically by measuring the absorption of a sample at 222.5 mμ, allowing for absorption by iodate ion. Using methyl-β-D-glycero-D-guloheptoryranoside as a standard compound, samples of freeze-dried chrisnose and crystalline heptose were dissolved in 0.015M sodium metaperiodate and the solutions incubated in the dark at room temperature for 8 hours. A periodate solution containing a drop of ethylene glycol was similarly incubated, and dilutions of all samples were measured for 222.5 mμ absorption. The results are shown below:-

$NaIO_4$ = 0.015M

weight of chrisnose = 6.0mg (in 20 ml IO_4')

Thus chrisnose soln. = 0.00139M (assuming M.wt. = 215)

weight of heptoside = 6.3mg (in 25 ml IO_4')

Thus heptose soln. = 0.00113M

Absorption readings of dilutions

$NaIO_4$ (B)	= 0.331
IO_3^- (C)	= 0.083
chrisnose solution (A)	= 0.298
heptoside solution (A)	= 0.268

The sugars thus consume periodate as follows.

chrisnose	1.44
heptoside	3.4 moles IO_4^- per mole of sugar

The results for chrisnose in particular are subject to errors on account of the small optical density changes which occurred on oxidation. Unfortunately there was insufficient time to repeat the measurements. In the discussion, the results are tentatively interpreted on the basis of the consumption of two moles of IO_4^-. The periodate consumption for chrisnose appeared to be unchanged after a further 16 hours.

b) Formic acid determination

In the periodate oxidation of sugars containing α, β, - triols one molecular proportion of formic acid is produced. Using the method of Barker and Somers (97), such formic acid is complexed with 2-thiobarbituric acid to give a chromophore of λ_{max} 450 mμ. The procedure adopted was exactly as in the literature except that the sodium borohydride was not buffered and that the butyl alcohol was not redistilled. In addition to the sodium formate used for calibration purposes, a sample of heptose methyl glycoside was also examined as in (a) above.

Results

weight of chrisnose = 3 mg in 1 ml periodate solution

weight of heptoside = 2 mg in 1 ml periodate

Formic acid produced

from chrisnose 600 units

from heptoside 398 units

From the calibration curve, 1 mole of chrisnose produces 0.93 moles of formic acid upon periodate oxidation, and 1 mole of heptoside produces 0.97 moles. (It is surprising that over-oxidation has not occurred for the latter.)

Note The report by Barker and Somers that acetic acid does not interfere with the analysis was confirmed. It is not known to what extent formyl esters produced by oxidation would be reduced to methanol in the assay, and to what extent they would interfere.

c) Formaldehyde determination

Periodate oxidation of terminal glycol groups results in formation of formaldehyde, which can be determined using the modification of the method by MacFadyen (98). Residual periodate is reduced using sodium sulphite (0.4M) and 0.5 ml samples containing about 30 heated at 100° for 30 minutes with chromotropic acid solution (1g sodium salt of chromotropic acid + 100 ml water + 400 ml of 25N sulphuric acid). After cooling, thiourea (4.6%, 2 ml) was added, and the optical densities read at 570 mμ.

For this experiment, absolute values were not obtained, relative values between formaldehyde from methyl-β-D-glycero-D-guloheptopyranoside and chrisnose instead being determined. From dilutions of the complexes obtained using 3.1 mg of chrisnose and 2.8 mg of heptose, the optical densities obtained were,

heptoside 3.92

chrisnose 0.41

Since the heptoside would produce 1 mole of formaldehyde per mole of sugar, it is concluded that chrisnose does not produce formaldehyde.

12. Colour reactions

The degradation products formed by carbohydrates in strong acid solutions may complex with suitable reagents to form chromophores characteristic of the type of carbohydrate, or more specifically, monosaccharide, present. Typical of such colour reactions are the phenol-sulphuric acid, L-cysteine/sulphuric acid diphenylamine/hydrochloric acid and carbazola/sulphuric acid tests.

Phenol/sulphuric acid test (61)

To 1 ml of chrisnose solution containing about 100 µg sulphuric acid (5 ml) was added in a steady stream from a fast-flowing burette, the test-tube being agitated during the addition. A blank containing no sugar was similarly treated, and after 30 minutes the reddish-brown sample solution was examined in the visible range on an automatic spectrometer. Literature and

experimental results are recorded below:

hexoses and methylated hexoses	490 mμ
pentoses, uronic acids and their methylated derivatives	480 mμ
chrisnose	485 mμ

L-cysteine/sulphuric acid test (99)

To 1 ml of chrisnose solution containing 50 μg was added, with cooling in ice, 5 ml of conc. sulphuric acid (5 parts AnalaR sulphuric acid + 1 part water). After 2 minutes, the sample was vigorously shaken and then heated in a boiling water-bath for 3 minutes. After cooling, L-cysteine hydrochloride solution (0.1 ml, 3% in water) was added and the mixture shaken. Absorptions in the visible range are as follows:

pentoses and hexuronic acids	390 mμ
hexoses	412 - 414 (→ 605 overnight)
heptoses	430
chrisnose	414 → 605 overnight

Diphenylamine/hydrochloric acid test (100)

The reagent was prepared by mixing 1 ml of a 10% alcoholic solution of diphenylamine with acetic acid (9 ml) and concentrated hydrochloric acid (10 ml). 2 ml of this reagent was added to 1 ml of aqueous sugar solution containing about 100 μg and the mixture heated at 100° for 25 minutes.

		λ
hexoses	blue colour	λ_{max} 520 and 635 λ_{min} 560
pentoses and tetroses	green/yellow	
hexuronic acids	brown/red	λ_{max} 520 - 522
heptose	purple	λ_{max} 560
chrisnose	blue	λ_{max} 522 and 646 λ_{min} 561

Carbazole/sulphuric acid test ( )

To 1 ml of chrisnose solution containing about 50 was added with cooling concentrated sulphuric acid (6 ml.) and the mixture was heated at 100° for 20 minutes. After cooling, 0.2 ml of a 0.1% ethanolic solution of carbazole was added and the solution left to stand for 2 hours.

Glucuronic acid standard gave a purple chromophore of λ_{max} 525 mμ while glucose comparison standard gave no chromophore apart from the slightly brown appearance of the solution during the heating with acid. Chrisnose and X failed to give the chromophore and their reaction was characterised by formation of a very dark brown solution during the acid treatment. It is appropriate to mention at this point that the uronic acid content of polysaccharides is determined in the Department of Bacteriology using the carbazole method. Chrisnose does not apparently interfere, and a result of about 18% has been obtained, a result which concords with our uronic acid analysis using the

decarboxylation method.

The colour reactions listed above thus indicate that the structure of chrisnose is such as to give the colour reactions characteristic of a hexose.

Triphenyl retrazolium chloride spray (102)

A small sample of chrisnose solution was applied to a filter-paper and allowed to dry. The paper was sprayed with 2% ethanolic 2,3,5-triphenyl retrazolium chloride containing an equal volume of 2N sodium hydroxide. Upon holding the paper over a boiling water-bath, a bright pink spot resulted. Such a positive reaction indicates that the hydroxyl groups on C_1 and C_2 are unsubstituted.

13. Electrophoresis

Electrophoresis of chrisnose showed that the sugar was immobile in the buffer employed.

Part D

P.m.r. spectroscopy of X glycoside indicates the presence of 5 methoxyl groups, one of these an ester methoxyl, and also indicates the probable presence of a C-methyl grouping. Further evidence that an ester grouping is present comes from the infra-red and optical rotatory dispersion experiments.

Using the assumption that some degradation occurs via the typical C_1-elimination pathway, a molecular weight of 278 has been assigned to the glycoside by mass spectrometry. A similar result has been obtained from vapour-pressure osmometry determinations and from elemental analysis estimations. The molecular weight estimations on chrisnose together with the p.m.r. interpretations for X-glycoside lead to the assumption that chrisnose has 4 hydroxyl groups in addition to an ester grouping.

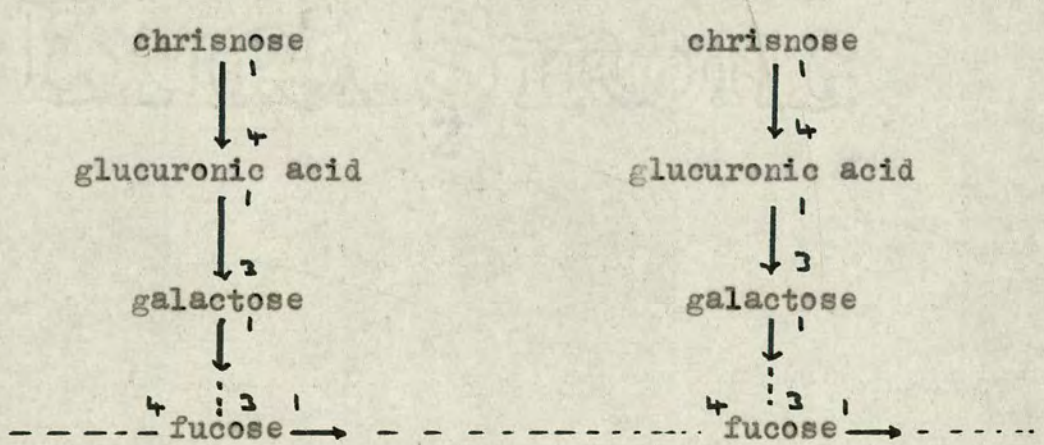
As stated in the experimental, the periodate results were rather indefinite, but periodate is consumed - about 2 moles - with the possible production of 1 mole of formic acid. Chrisnose may thus contain 3 contiguous hydroxyl groups, and the positive reaction of the sugar to the triphenyl tetrazolium chloride reagent would imply that these hydroxyl groups are on C_1, C_2 and C_3 of the molecule. Chrisnose behaves in colour reactions in a manner characteristic of hexoses rather than of heptoses.

The acid degradation product of chrisnose has a paper-chromatographic mobility similar to that of fucose. Difficulties encountered (Section 1) in the formation of a derivative of chromatographically separated fucose may have been caused by the presence of this impurity. The formation of an electrophoretically immobile product upon acid treatment of X glycosides is an unexpected result.

GENERAL SUMMARY

a) E.coli K.12 polysaccharides

Acid hydrolysis of the native and methylated polysaccharides of E.coli K.12 strains has revealed a striking basic constancy in the nature of the monosaccharide units and their positions of linkage. On the evidence presented in preceeding Sections, however, only very general conclusions can be drawn regarding the overall structure of these closely related molecules, and the "repeating unit", if it exists, is not likely to be simple. Partial structures elucidated give the polysaccharide the following, very incomplete structure:-



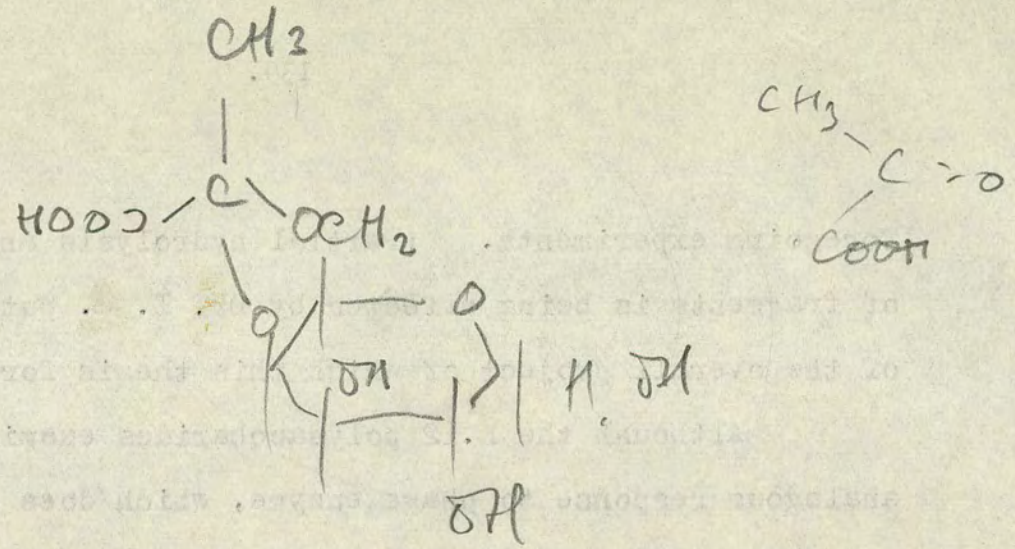
Unbranched 3-linked glucose units must be accommodated in the molecule, as must also a small proportion of main-chain glucuronic acid units if credence is given to the indications from alkaline fragmentation that these exist. However, the location of these units and other information necessary for fuller representation of the structure cannot be deduced from the results of

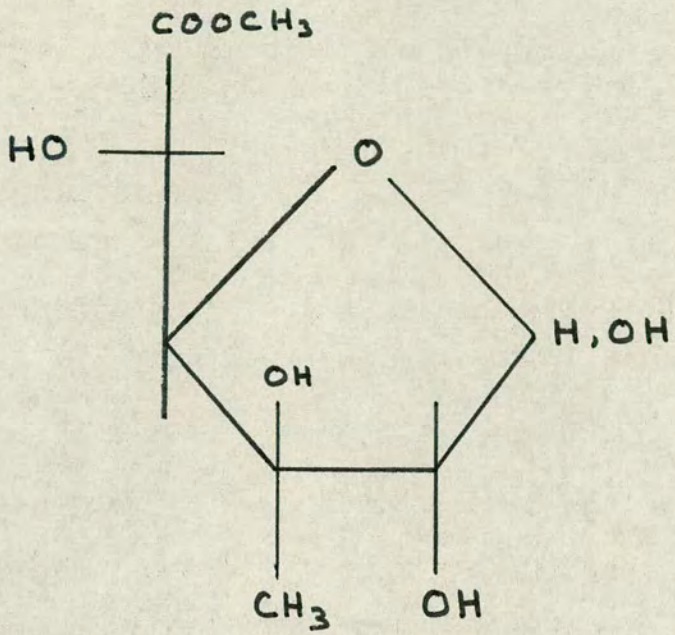
foregoing experiments. (Partial hydrolysis and identification of fragments is being effected by Dr. I. W. Sutherland as part of the overall project of which this thesis forms a part).

Although the K.12 polysaccharides examined show analogous response to phage enzyme, which does not appear to act peripherally on the molecules, the biological variation of these polysaccharides from slime to capsule, with variation in viscosity characteristics, is unexplained. Molecular size and shape, together with degree of esterification and acylation, are all variables which have not been considered in this thesis.

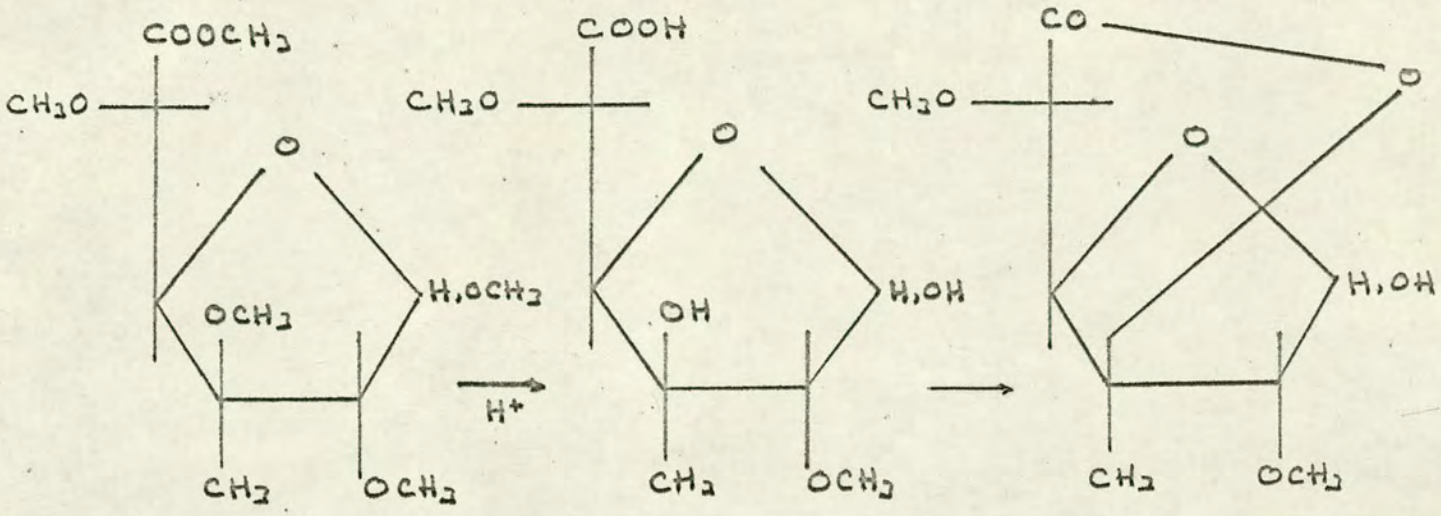
b) chrisnose

The use of controlled β-elimination on S53 polysaccharide under mild conditions has been of double importance in structural determination. Not only has the monosaccharide product, chrisnose, proved to be an unusual and probably unreported sugar, but this method have proved to be the only successful method of isolation of many that have been attempted. In the light of the results reported in the preceeding section, a provisional partial structure for chrisnose is postulated as





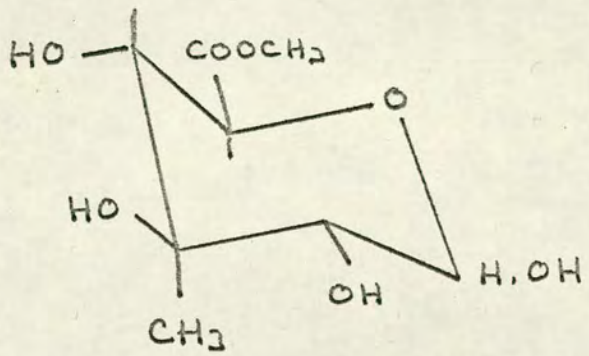
The D-gluco configuration is written for purposes of illustration, though there is little evidence for this. The methyl ester is drawn because this would be formed by MeOH/HCl treatment in the isolation of X, or CH_3O^- on another ester in the isolation of chrisnose. The results obtained in Section 6 can be rationalised in terms of this structure, which can also explain the lability of the sugar in acid. C_3 constitutes a tertiary alcohol, and acid lability is an inherent feature of such groupings. Acid treatment of methylated chrisnose (X), with formation of an electrophoretically immobile product, could be explained by lactone formation:-



The hydroxyl group is exposed for lactone formation by hydrolysis of the tertiary ether.

The furanose structure might explain the stability of X in β-elimination of methylated polysaccharides. Since X would have the same ring structure in the polymer, it would possibly be resistant under conditions which would destroy C_4- substituted glucuronic acid ester.

Assuming D-gluco stereochemistry, which is partly justified by apparent lactone formation, the tendency of chrisnose to exist as a furanose form might be explained by the instability imparted to the pyranose by an axial CH_3- group:-



GENERAL METHODS OF INVESTIGATION

Concentration of Polysaccharide Solutions. Solutions were concentrated under reduced pressure using a rotary-film evaporator with a bath temperature of 40°C or less.

Dialysis. Polysaccharide solutions were dialysed in cellophane tubes suspended in running tap-water. Chloroform was added to prevent spoilage.

Carbohydrate Contents. These were determined by the phenol/sulphuric acid method (89) using the procedure outlined in reference 61, page 388.

Complete Methanolysis. The material (1-10mg) was sealed in a tube with methanolic hydrogen chloride (approx. 3%) and heated at 100° for 4-6 hours. or at 70° overnight.

Methanolic Hydrogen Chloride. A 3% solution (w/v) was prepared by the careful addition of acetyl chloride (6 ml.) to methanol (100 ml.). The solution was shaken and kept for a few hours before using.

Melting Points. These were obtained on a Kofler hot-stage apparatus. Melting points are uncorrected.

Paper and Cellulose-column Chromatography. Whatman no. 1 or no. 4 paper was used for qualitative chromatography, Whatman 3 MM filter sheets being used for preparative paper chromatography.

The following solvent systems were used:

A) Ethyl acetate, pyridine, water 8:2:1

B) Ethyl acetate, acetic acid, formic acid water 18:3:1:4

C) Ethyl acetate, pyridine, water 10:4:3

D) Methyl ethyl ketone, acetic acid, water 9:1:1
(saturated with boric acid.)

E) Ethyl acetate, acetic acid, formic acid, water 18:8:3:9

F) Methyl ethyl ketone, water, ammonia (S.G.0.88) 200:17:1

G) Butan-1-ol, ethanol, water (upper layer) 4:1:5

H) Benzene, ethanol, water (upper layer) 169:47:15

The following detection sprays were used:

1) p-anisidine hydrochloride (90) (for reducing sugars). The dried chromatogram was sprayed with a 3% solution of p-anisidine hydrochloride in water-saturated n-butanol and heated at about 120°C for 10 minutes.

2) Alkaline silver nitrate (ref. 60, p.178) (for reducing sugars and glycol groups). A saturated aqueous solution of silver nitrate (0.1 ml) was diluted to 20 ml with acetone, and by the dropwise addition of water, the precipitated silver nitrate was re-dissolved. The dried chromatogram was quickly dipped in this reagent and dried. The chromatogram was then sprayed with 0.5N sodium hydroxide in aqueous ethanol. Excess background was removed with thiosulphate.

3) Periodate-benzidine (59) (for compounds containing glycol groups) The dried chromatogram was sprayed with an aqueous solution of sodium periodate (0.3% w/v) and left ten minutes to dry. The chromatogram was then dipped in a solution made by dissolving benzidine (184 mg) in a mixture of glacial acetic acid (0.6 ml), water (4.4 ml) and acetone (95 ml).

Gas-liquid Chromatography This was carried out using a Pye Argon chromatograph fitted with a strontium-90 detector, or on a Pye 104 chromatograph with hydrogen-flame detectors. Retention times (T) were calculated relative to the faster of the two methyl 2,3,4,6-tetra-O-methyl glucopyranoside peaks. Sample size varied but was usually of the order of 0.1µl. in chloroform solution. For the attempted preparative gas-liquid chromatography, Aerograph Autoprep was used. The following column packings were employed:

1) neopentyl glycol adipate - a 10% coating on 80/100 mesh celite
2) polyethylene glycol adipate - a 15% coating on 'Gas Chrom P'
3) SE-52 - a 3% coating on 'Gas Chrom P'.
4) XE 60 - a 3% coating on 'Gas Chrom P'.

Unless otherwise stated, columns were operated at 175°C, the gas-flow for the Pye Argon chromatograph being about 60ml/min.

Thin-layer Chromatography Qualitative experiments were carried out using micro-plates, and preparative experiments were done using large (20 x 20 cm) plates. Most of the thin-layer experiments described were done using a coating of silica gel (Kieselgel G, nach Stahl, Merck). The solvent systems used are quoted in the text. The detection of spots or bands was effected either by leaving the plate in a container saturated with iodine vapour for several minutes, or by spraying the plate with 4N sulphuric acid until just visibly damp and heating carefully over a low bunsen flame to develop the spots.

Optical Rotations These were measured using a Perkin-Elmer 141 automatic polarimeter, and employing the sodium D-line through a

1 dm. polarimeter tube. The solvent and concentration are quoted with the result.

Optical Rotary Dispersion This experiment was carried out using a Benderic Polarmatic 62 machine and employing spectroscopic alcohol as a solvent.

Proton Magnetic Resonance Spectra These were recorded using a Perkin-Elmer R 10 (60 Mc/sec) nuclear magnetic resonance spectrometer.

Methoxyl Contents and Elemental Analyses. These were performed by a commercial firm (A. H. Baird, Edinburgh, or Weiler and Strauss, Oxford) specialising in such analyses.

Ultra-violet Absorption Spectra These were recorded using a Perkin-Elmer 137 Spectrophotometer for qualitative investigations and a Unicam SP 500 for quantitative work.

Infra-red Absorption Spectra These were recorded using a Perkin-Elmer 237 spectrophotometer. Methylated polysaccharides were usually examined as thin solid films between sodium chloride plates.

Electrophoresis Electrophoresis was carried out in pyridine/acetic acid buffer (0.1M, pH 6.5) using a potential of 250 volts.

BIBLIOGRAPHY

1. STACEY and BARKER — "Polysaccharides of Micro-organisms", O.U.P. (1960)
2. ARCHIBALD and BADDILEY — Adv., 21, 323 (1966)
3. HOW, BRIMACOMBE and STACEY — Adv., 19, Table on pp.351-352 (1964)
4. MORGAN and ELSON — Biochem.J., 28, 988 (1934)
5. CRUMPTON and DAVIES — Biochem.J., 64, 22P (1956)
6. SHARON — Nature, 179, 919 (1957)
7. GHUYSEN and SALTON — Biochem. et. Biophys. Acta, 40, 462 (1960)
8. DAVIES — Nature, 180, 1129 (1957)
9. CRUMPTON and DAVIES — Biochem.J., 70, 729 (1958)
10. BARKER, BRIMACOMBE, HOW, STACEY and WILLIAMS — Nature, 189, 303 (1961)
11. DOY and GIBSON — Biochem.J., 72, 586 (1959)
12. LINGERNS and KERN — Z.physiol.chim. 318, 56 (1960)
13. KUHN and KIRSCHENLOHR — Ann., 600, 115 (1956)
14. STRANGE and POWELL — Biochem.J., 58, 80 (1954)
15. BARRY and GOEBEL — Nature, 179, 206 (1957)
16. DEWITT and ZELL — J.Bacteriol., 82, 838 (1961)
17. CORNFORTH, FIRTH and GOTTSCHALK — Biochem.J., 68, 57 (1958)
18. ROSEMAN and COMB — J.A.C.S. 80, 3166 (1958)
19. KUHN and BROSSMER — Ann., 616, 221 (1958)
20. MARKOWITZ — J. Biol. chem., 237, 1767 (1962)
21. MacLENNAN, SMITH and RANDALL — Biochem. J. 74, 3P (1960)
22. FROMME, NOWOTNY, LUDERITZ and WESTPHAL — Pharm. Acta. Helv, 33, 391 (1958)

23. SALTON — Biochim et. Biophys. Acta., 45, 364 (1960)

24. DAVIES — Nature, 191, 43 (1961)

25. FOUQUEY, POLONSKY, LEDERER, WESTPHAL and LUDERITZ — Nature, 182, 944 (1958)

26. LUDERITZ, WESTPHAL, STAUB and LeMINOR — Nature, 188, 556 (1960)

27. GORIN and SPENCER — Can. J. Chem. 44, 993 (1966)

28. CARLSON and MATTHEWS — Biochemistry N.Y., 5, 2817 (1965)

29. HEYNS and PAULSEN — Ber., 88, 188 (1955)

30. PERKINS — (Proc) Biochem. J., 89 (No.3), 104 (1963)

31. DAVIES — Adv., 15, 271 (1960)

32. SLEIN and SCHNELL — Proc. Soc. Exptl. Med., 82, 734 (1953)

33. MacLENNAN and DAVIES — Biochem. J., 66, 562 (1957)

34. WEIDEL — A.Physiol. chim, 299, 253 (1955)

35. DAVIES — Biochem. J., 63, 105 (1956)

36. ADAMS and YOUNG — Can. J. Chem., 43, 2929 (1965)

37. CLAUS — Biochem. Biophys. Res. Comm., 20, 745, (1965)

37a STOUTHAMER — Biochem. Biophys. Acta, 48, 484 (1961)

38. HEATH and GHALAMBOR — Biochem. Biophys. Res. Comm., 11, 288 (1963)

39. LEVIN and RACKER — J. Biol. Chem., 234, 2532 (1959)

40. OSBORN — Proc. Natl Acad. Sci. U.S., 48, 1542 (1962)

41. HEATH and EDSTROM — Biochem. Biophys. Res. Comm., 16, 576 (1964)

42. GHALAMBHOR, LEVINE and HEATH — J. Biol. Chem. 241, 3207 (1966)

43. PERRY — Can. J. Chem., 45, 1295 (1967)

44. STACEY and RICKETTS — Fortschr. Chem. Org. Naturforsch, 8, 28 (1951)

45. THOMA and STEWART — in "Starch, Chemistry and Technology" Vol. 1, 209, Acad. Press.

46. GORIN, SPENCER and WESTLAKE — Can. J. Chem, 39. 1067 (1961)

47. JOHNSON and CHILTON — Science, 152, 1247 (1966)

48. CONRAD and SANDFORD — Biochem., 1508 (1966)

49. CONRAD et al — ibid, 2808

50. ASPINALL, JAMIESON and WILKINSON — J.C.S., 3483 (1956)

51. GAREGG and LINDBERG — Acta. Chem. Scand., 14, 338 (1960)

52. SUTHERLAND — Biochem. J., in press

53. HOTCHKISS and GOEBEL — J. Biol Chem., 121, 195 (1937)

54. JONES and PERRY — J. Am. Chem. Soc., 79, 2787 (1957)

55. BARKER, HEIDELBERGER, STACEY and TIPPER — J. Chem. Soc., 3468 (1958)

56. KABAT et al — J. Exptl. Med., 88, 43 (1948)

57. PALMER and GERLOUGH — Science 92, 155 (1940)

58. HEIDELBERGER et al — J. Exptl. Med., 46, 601 (1927)

59. GORDON, THORNBURG and WERUM — Anal. Chem., 28, 849 (1956)

60. BLOCK, DURRUM and ZWEIG — "A Manual of Paper Chromatography and Electrophoresis" 2nd Edn. Acad. Press. N.Y.

61. WHISTLER and WOLFROM — "Methods in Carbohydrate Chemistry". Vol. 1.

62. EASTERBY, HOUGH and JONES — J. Chem. Soc., 3416 (1951)

63. BELL — J. Chem. Soc., 1461 (1947)

64. HIRST, JONES and WOODS — J. Chem. Soc., 1048 (1947)

65. MANDL and NEUBERG — Arch. Biochem. Biophys, 35, 326 (1952)

66. OWEN, PEAT and JONES — J. Chem. Soc., 339 (1941)

67. WILSON — Anal. Chem. 31, 1199 (1959)

68. ANDERSON — Talanta, 2, 73 (1959)

69. PEAT, WHELAN and ROBERTS — J. Chem. Soc., 2258 (1956)

70. BAILEY — "Oligosaccharides" Pergammon Press, 1965.

71. VAN DER VEEN — J. Org. Chem., 28: 564 (1963)

72. RODEN and MARKOVITZ — Biochim. Biophys. Acta 127, 252 (1966)

73. ASPINALL and CANAS-RODRIGUEZ — J.Chem. Soc., 4020 (1958)

74. SRIVASTAVA — Tetrahedron Letters, 10, 493 (1964)

75. CROSS — "Practical Infra-red Spectroscopy" 2nd Edition, 69.

76. CONCHIE and PERCIVAL — J. Chem. Soc., 2573 (1950)

77. HAWORTH and SEDGEWICK — J. Chem. Soc., 2573 (1926)

78. ASPINALL, JAMIESON and WILKINSON — J. Chem. Soc., 3483 (1956)

79. SMITH — J. Chem. Soc., 1035 (1940)

80. HORECKER — Ann. Rev. Microbiol., 1966

81. RICHARDS and SEPHTON — J. Chem. Soc., 4492 (1957)

82. NEUKOM and DEUEL — Chem. and Ind., 36, 683 (1958)

83. KENNER — Chem. and Ind., 727 (1957)

84. REES and RICHARDSON — Biochemistry, 5, 3099 (1966)

85. BARRETT and NORTHCOTE — Biochem. J., 94, 617 (1965)

86. PREISS and ASHWELL — J. Biol. Chem., 237, 309 (1962)

87. NAKADA and SWEENY — J. Biol. Chem., 242, 845 (1967)

88. LINKER, MEYER and HOFFMAN — J. Biol. Chem., 219, 13 (1956)

89. DUBOIS et al — Anal. Chem., 28, 350 (1956)

90. PRIDHAM — Anal. Chem., 28, 1967 (1956)

91. WILKINSON and SUTHERLAND J. Gen. Microbiol, 39 373 (1965)

92. KOCHETKOV and CHIZHOV Adv., 21 (1966)

93. KUHN and TRISCHMANN Chem. Ber., 96, 284 (1963)

95. BHATTERCHARJEE Ph.D. Thesis, Univ. of Edin. 1967

96. ASPINALL and FERRIER Chem. and Ind., 1216 (1957)

97. BARKER and SOMERS Carb. Res., 3, 220 (1966)

98. MACFADYEN J. Biol. Chem. 158, 107 (1945) modification by Annan, Ph.D. Thesis, Univ. Of Edin.

99. WHISTLER and WOLFROM (Eds) "Methods in carbohydrate chemistry" Vol. 1, Acad. Press. N.Y. p. 488

100. Ibid. p. 491

101. Ibid. p. 497

102. Ibid. p. 513